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Elucidating the Targets and Function of the MLR Compass-Like Complex During Development

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ELUCIDATING THE TARGETS AND FUNCTION OF THE MLR COMPASS-LIKE COMPLEX DURING DEVELOPMENT

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To the flies that lived and died to better our understanding.

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LIST OF ABBREVIATIONS

3'UTR	3' Untranslated Region
Akh	Adipokinetic Hormone
AMP	Antimicrobial Peptide
ASH2L	Set1/Ash2 histone methyltransferase complex subunit ASH2
BG	Blue Gut
β-Gal	β-Galactosidase
bantam OE	*bantam* Overexpression
*ban*sens-GFP	*bantam* Sensor GFP
*ban*sponge	*bantam* Sponge
bee	*bantam* Eye Enhancer
Bmm	Brummer
BMP	Bone Morphogenetic Protein
Brat	Brain Tumor
Brk	Brinker
bwe	*bantam* Wing Enhancer
cDNA	Complementary DNA
CG	Clear Gut
ChIP-seq	Chromatin Immunoprecipitation Sequencing
Cmi	Cara Mitad
Cmi KD	Cmi Knockdown
Cmi OE	Cmi Overexpression
COMPASS	Complex of Proteins Associated with Set1
CTCF	CCCTC Binding Factor
Dcp-1	Drosophila Caspase 1
DE-Gal4	Dorsal Eye Gal4
Dif	Dorsal-related Immunity Factor
Dl	Dorsal
DNA	Deoxyribonucleic Acid
Dpp	Decapentaplegic
Dpy30	Protein dpy-30 homolog
dUTP	Deoxyuridine Triphosphate
Ec	Ecdysone
EcL	Eclosion Lethal

EcR	Ecdysone Receptor
EGFP	Enhanced GFP
EGFR	Epidermal Growth Factor Receptor
Ena	Enabled
En-Gal4	Engrailed Gal4
ER	Estrogen Receptor
Eyg	Eyegone
Ey-Gal4	Eyeless Gal4
Foxo	Forkhead Box Protein O
Foxo KD	Foxo Knockdown
Foxo OE	Foxo Overexpression
FXR	Farnesoid X Receptor
GCR	Glucocorticoid Receptor
GFP	Green Fluorescent Protein
GMR-Gal4	Glass Multiple Reporter Gal4
Gnmt	Glycine N-methyltransferase
H_2O_2	Hydrogen Peroxide
H3K4me	Histone 3 Lysine 4 Methylation
H3K27ac	Histone 3 Lysine 27 Acetylation
H3K27me	Histone 3 Lysine 27 Methylation
Hes1/4	Hairy and Enhancer of Split 1/4
Hh	Hedgehog
Hid	Head Involution Defective
HOX	Homeobox
HMG	High-Mobility Group
Hth	Homothorax
Ilp	Insulin-like Peptide
Ilp6	Insulin-like Peptide 6
IPC	Insulin Producing Cell
Jag1/2	Jagged 1/2
Jhamt	juvenile hormone acid methyltransferase
kb	Kilobase
KD	Knockdown
lncRNA	Long Non-coding RNA
Lsp2-Gal4	Lipid Serum Protein 2 Gal4
Mad	Mothers Against Dpp
MAPK	Mitogen-Activated Protein Kinase
Mbp	Mega-basepair
miRNA	Micro RNA

mg	Milligram
mL	Milliliter
MLR	MLL/Trr
MLX	MLL/Trx
mRNA	Messenger RNA
NCoR	Notch Corepressor
OE	Overexpression
Omb	Optomotor Blind
PBS	Phosphate-Buffered Saline
PBST	PBS Triton X-100
PBSTB	PBST Fetal Bovine Serum
PCR	Polymerase Chain Reaction
PGR	Progesterone Receptor
PHD	Plant Homeodomain
PI3K	Phosphatidylinositol 3-kinase
PL	Pharate Lethal
PPARγ	Peroxisome Proliferator-activated Receptor Gamma
PRC2	Polycomb Repressor Complex 2
Pros	Prospero
RbBP5	Retinoblastoma-binding protein 5
RBPJ	Recombination Signal Binding Protein for Immunoglobulin Kappa J Region
RAR	Retinoic Acid Receptor
RAS	Ras GTPase
RNA	Ribonucleic Acid
RNAi	RNA Interference
RNA pol II	RNA Polymerase II
RNA-seq	RNA Sequencing
Sd	Scalloped
SdBP	Scalloped Binding Protein
SEM	Scanning Electron Microscopy
siRNA	Small Interfering RNA
Su(h)	Suppressor of Hairless
TAG	Triglyceride
TdT	Terminal Deoxynucleotidyl Transferase
TGF-β	Tumor Growth Factor β
Thor	Thor (4E Binding Protein)
Trbl	Tribbles
Trr	Trithorax Related
Trr KD	Trr Knockdown

Trx	Trithorax
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
UAS	Upstream Activating Sequence
Utx	Ubiquitously Transcribed X-linked
Wdr5	WD repeat-containing protein 5
Wg	Wingless
Wg^{sp-1}	Wingless Sternopleural Mutation
WTT	White-eyed, TM3, TM6B
Yki	Yorkie

CHAPTER 1

INTRODUCTION

MLR COMPASS-like complexes are highly-conserved epigenetic regulators required for enhancer establishment and subsequent reprogramming during differentiation and development. Mutation of MLR complex subunits in humans is associated with cancer and developmental disorder, yet much remains to be determined concerning both the healthy and disease-altered functions of these complexes. Using the developmental model *Drosophila melanogaster*, I further elucidate the functions of the MLR complex during *in vivo* organ development as well as stress response. I characterize the miRNA *bantam* as a regulatory target of the complex, required for proper tissue patterning during wing and compound eye formation. In the same systems I confirm *in vitro* evidence that the MLR complex is required to establish enhancers for regulatory activity cell generations before reprogramming, and further demonstrate a protective role against apoptosis in undifferentiated tissue. Using the fat body as a model of metabolic activity and stress response, I demonstrate that the depletion rate of triglyceride stores during nutrient stress is sensitive to MLR complex activity, and suggest that this is an indirect effect of the regulation of stress response signaling pathways. Through these investigations I demonstrate that the MLR complex may function to either promote or suppress the activity of a single transcriptional effector or the transcription of a single regulatory target, depending on the contexts of development and cell fate.

CHAPTER 2

LITERATURE REVIEW AND BACKGROUND

Enhancers are Distal Regulatory Elements

The development of large multicellular organisms from a single zygote involves proliferation and development into millions to trillions of specialized cells of diverse form and function, determined by differential gene expression from a single genome. These processes require complex spatio-temporal patterns of developmental signaling that reprogram gene expression during multiple periods of differentiation and cell fate determination. The basic transcriptional unit, consisting of transcription factors binding to a promoter region and controlling initiation and elongation of transcription by RNA pol II, is inadequate for the fine control required to precisely respond to the suite of signals governing differentiation and reprogramming. Instead, expression of developmentally-regulated genes is controlled by cohorts of distal regulatory elements, also known as enhancers [1,2].

An enhancer consists of a short region of non-protein coding DNA harboring multiple transcription factor binding sites located up to 1Mbp either upstream or downstream from its target gene(s) [3,4]. Upon full enhancer activation, the enhancer is brought in contact with a target promoter through mechanisms that involve chromatin looping and insulation machinery such as Cohesin complex and CTCF that are not yet completely understood. Direct contact allows the enhancer to deliver multiple bound factors to the promoter including transcription

factors, RNA pol II, and the multi-subunit mediator complex; it is currently thought that this delivery of transcription promoting machinery is the main method by which enhancers stimulate activity of their targets. In general, a core promoter lacking enhancers' influence has stuttered transcription initiation and inefficient transcription elongation [1]. Promoter regions may be regulated by multiple enhancers, and a single enhancer may regulate multiple promoters. In essence, the complex web of enhancer-promoter communication is a network that translates the pattern of inputs from multiple transcription factors into precise regulatory decisions.

Activation of an enhancer is a multi-step process comprising changes in chromatin structure associated with post-translational histone modification [1,2]. A silent enhancer is composed of compact chromatin decorated with repression-associated histone marks such as trimethylation of the 27 lysine of histone 3 (H3K27me3). Pioneer transcription factors are the first to bind to silent enhancers, recruiting chromatin remodeling factors and histone modifiers that loosen the chromatin environment. This includes the deposition of activity-associated monomethylation of the 4 lysine of histone 3 (H3K4me1) and the replacement of repressive H3K27me3 with activating acetylation (H3K27Ac). Enrichment of H3K4me1 and H3K27Ac are hallmarks of active enhancers regulating gene expression. However, there are two intermediate stages of enhancer activation relevant for reprogramming: "primed" and "poised" enhancers [1]. Primed enhancers are enriched for H3K4me1 but lack both repressive methylation and activating acetylation of H3K27. While not as potent as fully-active enhancers, primed enhancers may still drive basal levels of target gene expression. Poised enhancers are more developmentally relevant and are critical for proper gene regulation following differentiation

and reprogramming. Poised enhancers contain both activity-associated H3K4me1 and repression-associated H3K27me3. In this state, these regions are not currently regulating transcription of target genes. However, as compared to fully-silenced and compact enhancers, poised enhancers are able to rapidly respond to appropriate regulatory stimuli and fully activate through replacement of H3K27me3 with acetylation. This ability is vital for the expedited yet precise reprogramming necessary during cellular differentiation. Consequently, pluripotent and multipotent undifferentiated cells are enriched for these poised enhancers; these sequences will either be fully activated or fully silenced accordingly during the processes of terminal cell fate determination. While a variety of molecular machinery is necessary for enhancer regulation, the establishment and activation of enhancers requires the activity of the MLR family of COMPASS-like complexes.

MLR COMPASS-like Complexes

COMPASS (COMplex of Proteins Associated with Set) and COMPASS-like complexes (previously known as ASCOM complexes) are ancient and highly-conserved epigenetic co-regulators responsible for H3K4 methylation (H3K4me) [5,6]. Yeast species contain one such complex responsible for all H3K4me, designated the Set1 complex after methyltransferase subunit Set1. Larger and more complicated eukaryotes contain multiple orthologous complexes with distinct H3K4 methylation activity and genomic targets. Each consists of 7-9 subunits, many of which are common among orthologous complexes, but some which are distinct to a family or individual complex. The most notable of these subunits are the methyltransferases, each of which is unique to a particular COMPASS-like complex (**Fig. 1**).

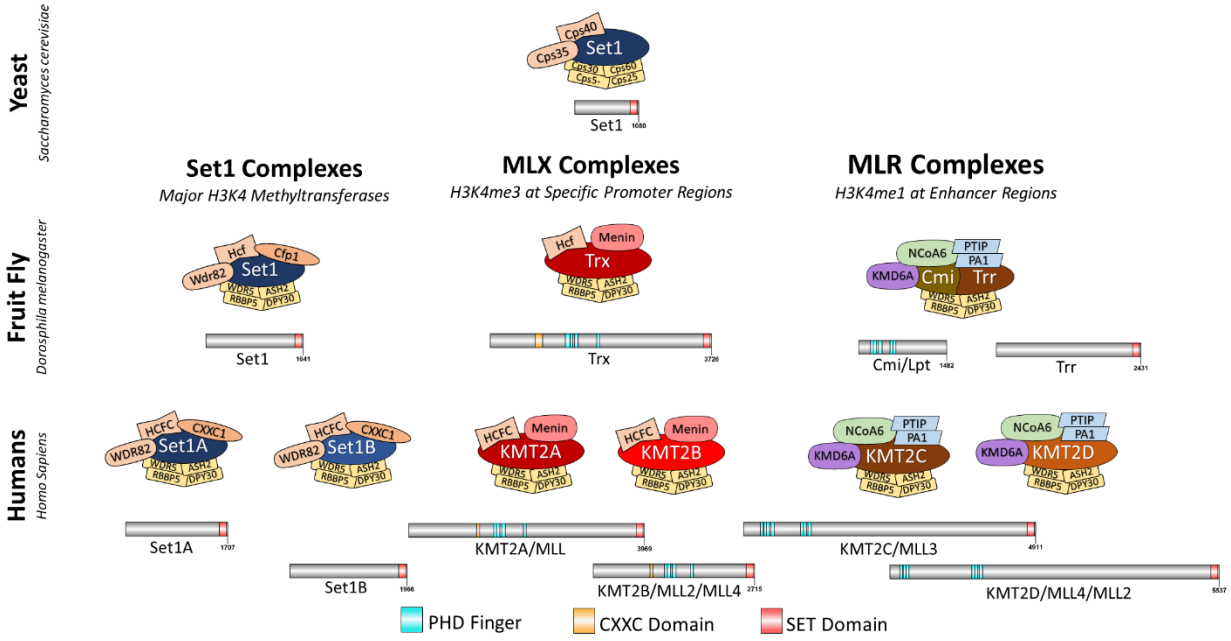

Figure 1. Orthology of COMPASS-like Complexes. COMPASS and COMPASS-like complexes are responsible for the deposition of H3K4me. Simple eukaryotes like yeast have a single complex, whereas more complex multicellular eukaryotes have multiple paralogous complexes, each with a unique methyltransferase subunit. The Set1 family of complexes are the major H3K4 methyltransferases, MLX complexes deposit H3K4me3 at Hox gene promoters and other specific regions, and MLR complexes deposit H3K4me1 at enhancer regions.

In contrast to the universal H3K4me activity of the Set1 complex in yeast, the multiple specialized complexes in higher organisms are required for proper development. The fruit fly, *Drosophila melanogaster*, harbors three such COMPASS-like complexes. These include the Set1 (responsible for the majority of H3K4me) [7,8], Trx (co-regulator of Hox-genes) [9], and Trr/Cmi (results of genetic split; localize to and regulate enhancers) [10,11] complexes. Humans and other mammals contain multiple orthologous complexes separated into three functional groups mirroring those in the fly, each with two partially-redundant paralogs [12]. SET1 and SET2 perform the vast majority of H3K4me; the more specialized COMPASS-like complexes and their titular methyltransferase subunits are referred to as KMT2(lysine[K] Methyl-Transferase 2) A-D. KMT2A (MLL1) and KMT2B (MLL4/MLL2), orthologous to *Drosophila melanogaster* Trx, are

necessary for regulating a variety of specific genetic targets, most notably Hox genes [13,14]. KMT2C (MLL3) and KMT2D (ALR/MLL2/MLL4), similar to both Cmi and Trr in flies, regulate enhancer sequences and deposit H3K4me1 [15,16]. We refer to these two families of COMPASS-like complexes as MLX complexes (orthologous to Trx, KMT2A, KMT2B) and MLR complexes (orthologous to Trr/Cmi, KMT2C, KMT2D) [17]. MLX complexes have been intently studied due to their roles in developmental patterning (via regulation of HOX genes) and alteration in human leukemias (gene fusion in mixed lineage leukemias, from which the MLLs derive the title) [18,19]. However, research from the last decade has demonstrated that MLR complexes are more critical for organismal development and mutation of these more significantly associated with oncogenesis [20].

MLR COMPASS-like complexes are recruited to enhancer regions by a variety of transcription factors, where they function by depositing activity-associated H3K4me1, removing repressive H3K27me3, and recruiting acetyltransferase p300/CBP (responsible for H3K27ac) [21–23]. They are widely regarded as epigenetic histone modifying tools brought in to activate enhancer regions and thereby regulate transcription. Recently, reports by our group and others have challenged the simplicity of this model and further elucidated the multifaceted roles that MLR complexes play during organismal development.

Components of MLR Complexes

MLR complexes each contain either nine or ten distinct protein subunits; paralogous complexes within a single species share all subunits excepting the methyltransferase, unique to each complex. Included in these is the WRAD complex (named for its four subunits WDR5, RbBP5, Ash2L, and Dpy30), common to all COMPASSS/COMPASS-like complexes and required

for stable methyltransferase activity [12]. The remaining subunits are exclusive to MLR COMPASS-like complexes. NCoA6 (Nuclear receptor Co-regulator A6) is a transcriptional coactivator known to associate with a variety of transcription factors, most notably nuclear receptors [24]. PTIP and PA1 also interact with various chromatin-binding proteins and aid in recruitment of the complex; the two have also been identified as forming a subcomplex with identified gene regulation activity separate from MLR [25,26]. KDM6A/Utx is an H3K27-specific demethylase, responsible for removing repressive H3K27me3 in tandem with deposition of activity-associated H3K4me by the methyltransferase [27,28] .

The central methyltransferase subunits themselves are remarkably large proteins of approximately 5,000 amino acids. The N-terminal halves of these proteins contain two clusters of PHD finger domains, necessary for stable and selective histone binding, as well as an HMG box that may harbor nucleic acid-binding activity. The C-terminal halves terminate in a SET domain, which harbors the enzymatic activity. Due to a likely ancestral gene-splitting event, Drosophila and other dipteran insects contain two separate genes individually coding for the N-terminal and C-terminal halves of the MLR methyltransferase. These are called Cmi/Lpt and Trr, respectively, and there is no current evidence that suggest that this split has altered the function of the Drosophila MLR complex relative to those in other species. In fact, due to the ancient nature of MLR complexes, there is high conservation of structure, function, binding partners, and regulatory targets among MLR complexes in all metazoans.

MLR Complexes are Necessary for Regulating Organismal Development

Initial study of the developmental role of MLR complexes occurred via whole-animal knockout of the methyltransferases in *Drosophila*. *Trr* deficient flies fail to complete

embryogenesis, while *Cmi* null animals suffer lethal developmental arrest at the second instar larval stage [10,11]. Loss of either *Kmt2c* or *Kmt2d* in mice during gestation results in lethality perinatally or during early embryogenesis, respectively [29]. Interestingly, loss of Kmt2c methyltransferase activity during development via targeted mutation results in living animals displaying inhibition of white adipose tissue development [30]. These results were the first indication that some MLR complex activity may be independent of the deposition of H3K4me1. Targeted knockout of *Kmt2d* in muscle and fat precursor cells resulted in mice that died shortly after birth, with severely underdeveloped musculature and adipose tissue. Murine brown pre-adipocytes deficient in both *Kmt2c* and *Kmt2d*, when induced to differentiate, demonstrate severely reduced adipogenesis and myogenesis potential and are unable to induce cell type-specific genes. In accordance with this, Kmt2d interacts with fat and muscle lineage-specific transcription factors and is necessary for properly priming critical differentiation-associated enhancer regions [29].

Intriguingly, rather than suggesting that MLR complexes are particularly associated with fat and muscle development, further studies have established that they play similar roles during differentiation into a wide variety of cell types: MLR associates with essential lineage-determining factors, binds to identified tissue-specific enhancer elements, and positively regulates the differentiation process of many cell lineages. In addition to interacting with C/EBPα/β and PPARγ during adipocyte differentiation and MyoD in myocytes [29], MLR methyltransferases have been found to associate with and regulate the targets of GrhL3 during epidermal differentiation [31], HOXA1-3 and Nestin during neuronal differentiation [32], ER during mammary gland formation [33], MafA and MafB during β-Cell development [34], NF-E2 during

erythroid and megakaryocyte development [35], Foxp3 during Treg lineage determination [36], p63 during epithelial developmental [37], Runx1 and Runx2 during osteogenesis [38,39], and Shox2 during chondrogenesis [40]. KMT2D is also necessary for cardiomyocyte-specific gene expression during heart development, although involved binding partners haven't yet been identified [41]. These studies suggest that MLR complexes are directly recruited to the regulatory targets by these transcription factors; those that do not verify physical interaction instead demonstrate that presence of the lineage-specific factors is required for MLR complex binding and activity. Significantly, many of these investigations determined that activity and viability of the pre-differentiated cells remains stable upon loss of MLR complex activity while only differentiation potential is inhibited. Even in pluripotent cells, *Kmt2d* loss inhibits differentiation timing and capacity but has no effect on self-renewal [21,42]. In summation, these current data suggest that MLR complexes are disposable for the normal function of cells, yet are required during transcriptional reprogramming.

While it has long been assumed that the methyltransferase activity is essential for MLR complex function, recent reports have demonstrated that H3K4me1 deposition is at least partially superfluous to in establishing enhancers and facilitating reprogramming [43,44]. Loss of enzymatic activity even allows for the development of a viable adult Drosophila [45]. Thus, the non-enzymatic functions of the MLR complex, such as presence at enhancers and cofactor recruitment, may be the more critical aspects of its function. Even given this, it is unlikely that such an ancient and conserved function as H3K4 monomethylation is not significant to the role of the complex. Evidence suggests that loss of enzymatic activity deleteriously affects certain tissues compared to others and can shift cell fate decisions during differentiation [30,46]. Current

data suggests that the roles of H3K4me1 as well as MLR complexes in general during development may be more subtle than previously assumed.

Recent results from our lab have revealed that in addition to being required to establish enhancers during early commissioning, the Drosophila MLR complex plays a role in bookmarking, remaining at poised enhancers until full activation. Consequently, the complex functions not only to allow eventual activation of enhancers, but also prevents premature activation before the appropriate developmental timepoint [47]. These results further elucidate the detrimental effects of loss of MLR complex activity.

Signaling Pathway Effectors and Transcription Factors Recruit MLR Complexes

In addition to interacting with and co-regulating the targets of specific lineage-determining factors, MLR complexes are necessary coactivators of highly-conserved developmental signaling pathways. Multiple studies have demonstrated that loss of complex function during organ development results in phenotypes similar to those associated with alteration of these pathways.

Hippo signaling. The Hippo pathway regulates cell growth to control organ size during animal development, which is accomplished through inhibition of transcription factor Yap. The Trr MLR complex is implicated as a co-regulator of Hippo signaling by its physical interaction with Yki (*Drosophila* Yap ortholog) and HCF (Hippo co-regulator), significant colocalization on chromosomes, and a positive regulatory role on the transcription of Yki target genes [48–50]. Depletion of components of the complex within developing tissues resulted in smaller adult organs with patterning defects, phenotypes similar to the inhibition of Yki activity.

TGF-β/Dpp signaling. The TGF-β/BMP paracrine signaling pathway regulates tissue patterning and development, consisting of the secretion of a short-range signaling molecule which binds to and activates nearby cell surface receptors, propagating a signal through Smad activation to the nucleus. Trr loss of function enhances the reduction of Dpp (Drosophila TGF-β ortholog) expression in developing organs [51] and transcription of Dpp requires the MLR complex [52]. This positive regulatory relationship is conserved in mammalian TGF-β signaling [53].

Wnt signaling. Wnt signaling is another highly-conserved tissue patterning paracrine/autocrine pathway involving the signal propagation from a ligand-bound cell surface receptor signal to the nucleus. Canonical Wnt signaling terminates in the nuclear translocation and activity of its effector protein, β-catenin. The transcription factor PITX2, target of β-catenin and downstream effector of Wnt signaling, interacts with the KMT2D MLR complex, which is necessary for its transcriptional activity [54].

Notch signaling. Notch juxtacrine signaling controls cell fate during development; it is accomplished through the cleavage of the transmembrane Notch receptor upon cell-cell contact ligand binding, the intracellular domain of which translocates to the nucleus and regulates transcription. Loss of Trr or Utx leads to increased Notch activity in developing tissues and suppresses the phenotypic effects of the loss of Notch function [55,56]. However both murine and Drosophila MLR complexes interact with RBPJ, a Notch binding partner, in order to compete for Spen/SHARP binding with NCoR (Notch CoRepressor) and positively regulate Notch target genes [57]. In the zebrafish, *kmt2d* is required to suppress *rbpj* expression during heart development, ensuring proper organ formation [58]. While MLR complexes have been demonstrated to be necessary for positively regulating the targets of other developmental

signaling pathways, current evidence suggests that they may function to either positively or negatively regulate Notch signaling in different contexts.

Nuclear receptor signaling. MLR complexes interact with and are necessary for regulating the targets of multiple hormone receptors across species, including ecdysone receptor (EcR) in Drosophila and estrogen receptor (ER) in mammals [33,51,59]. This regulatory relationship is not limited to steroid hormone signaling. Rather, MLR complexes have been found to interact with farnesoid X receptor (FXR) to regulate bile acid homeostasis [60,61], glucocorticoid receptor (GCR) during fat tissue development [62], progesterone receptor (PGR) to regulate progesterone targets [63], and retinoic acid receptor (RAR) in regulation of its transcriptional targets [64].

MLR Methyltransferase Germline Mutation is Associated with Developmental Disorders

Correct function of enhancer regulation machinery is critical during development, and alterations to this machinery, if survivable, often lead to developmental disorder and disease [65]. Histone lysine methyltransferases and demethylases are frequently mutated in such disorders, commonly due to haploinsufficiency of the associated genes [66]. Mutations in KMT2C and KMT2D, as well as in other subunits specific to MLR complexes, have been identified as causal events in Kleefstra Spectrum Syndrome and Kabuki Syndrome, respectively.

Kabuki syndrome. Kabuki syndrome is a phenotypically heterogenous congenital disorder named after the cranio-facial developmental abnormalities that cause resemblance to traditional Kabuki theatre masks, including distinct eye shape, dense eyebrows, prominent ears, and downturned corners of the mouth [67,68]. In addition, those suffering from this syndrome often experience developmental and growth delay, intellectual disability, and cardiac defects,

the latter of which are the main cause of mortality. The majority of those diagnosed with Kabuki syndrome harbor heterozygous inactivating germ-line mutations in KMT2D of MLR demethylase KDM6A [69–71].

Zebrafish models of Kabuki involving depletion of kmt2d or kdm6a verify the complex's role in brain, heart, and craniofacial development, and suggest that alteration of MAPK signaling and Notch signaling may underlie some of the resulting phenotypes [58,72,73]. Chemical agents targeting epigenetic machinery can be used to rescue neurodefective phenotypes in a Kabuki model, suggesting that further elucidation of MLR complex target and activity can be leveraged therapeutically [74].

Kleefstra syndrome. Kleefstra syndrome (previously known as 9q34 deletion syndrome) is a rare disorder, with less than 200 individuals definitively diagnosed. The true prevalence of this disease is difficult to determine, as only recently has genetic testing been able to distinguish it from developmental disorders with similar presentation. These symptoms include craniofacial abnormalities, significant developmental and intellectual disability, hypotonia, malformations of the brain, heart, and genitourinary system. Socially, autism-like behavior is common. The main genetic root of Kleefstra Syndrome is the deletion or inactivation of one copy of *EHMT1* (eukaryotic histone-lysine N-methyltransferase 1), a H3K9-specific methyltransferase [75]. Multiple Kleefstra patients lacking *EHMT1* mutation were found to harbor deleterious mutations in *KMT2C*; it was verified in Drosophila that KMT2C/Trr interacts with EHMT1/G9a during gene regulation, notably during memory formation [76,77].

MLR Methyltransferase Somatic Mutation is Associated with Cancer

Over the past two decades, a multitude of high-throughput human cancer genome and exome sequencing studies continue to identify *KMT2C* and *KMT2D* as among the most commonly mutated genes in a wide variety of tumor types with typical mutation frequencies of 10-40% [20]. Many of the identified mutations are nonsense and therefore cause loss of the C-terminal enzymatic SET domain, as well as potentially reducing methyltransferase levels due to nonsense-mediated degradation [78] or decreased protein stability [79]. Cancer-enriched missense mutations are localized to splicing sites and putative protein interaction sites as well as the PHD finger and SET domains [71]. As copy-number analyses become more common, amplification or deletion of either *KMT2C* or *KMT2D* have been significantly identified as well. The identified malignancies share very few characteristics besides cancerous growth and many of these reports have identified these as likely driving mutations of oncogenesis, suggesting characterization of *KMT2C* and *KMT2D* as classical tumor suppressors. However, unlike other frequently mutated tumor suppressors such as *p53* and *RB1*, it remains unclear how MLR complex alteration leads to malignancy.

As MLR complexes are critical co-regulators of cell fate transition, it's plausible that mutation leads to inhibition of differentiation and/or retention of a multipotent-like state, promoting or facilitating transformation. If so, it would be expected that the timing of MLR loss would determine oncogenic potential. Indeed, *KMT2D* deletion early in B cell development leads to induction of lymphoma, whereas loss after differentiation and germinal center formation does not [80,81]. In addition to regulating differentiation, the complexes have demonstrated a role in maintaining genomic stability. *KMT2D* loss in human or mouse cell lines

causes severe genome instability and transcriptional stress, leading to increased mutation rates [82], while downregulation of *KMT2C* reduces levels of DNA repair machinery and inhibits double-strand break repair [83]. KMT2C has also been shown to be recruited to replication forks by p53, and loss of this interaction directly leads to chromatin instability [84]. In fact, MLR complexes have a complicated relationship with both wild type and mutant p53. KMT2C/D have been repeatedly identified as coactivators of p53 transcriptional targets, and the loss of both methyltransferases in mammalian cells suppresses expression of these targets [61,85,86]. Conversely, certain p53 gain-of-function mutants upregulate *KMT2D* [87] and KMT2D acts as a co-activator of oncogenes regulated by mutant p53 [88], potentially resulting in a positive regulatory loop greatly increasing mutant p53 oncogenic activity in the cell. Differentiation inhibition combined with chromatin instability and downregulation of p53 targets has clear oncogenic potential. Of course, MLR complexes may also have undiscovered regulatory targets whose dysregulation promotes tumorigenesis. In a planarian tissue regeneration study, knockdown of Cmi and Trr leads to aberrant, unregulated cell growth and tumor-like outgrowths resulting from failure to differentiate; gene ontology analysis revealed an upregulation of cell division and proliferation genes and downregulation of cell differentiation and metabolism genes [89]. It remains unclear whether these targets are conserved in humans.

It is likely oversimplification to label *KMT2C* and *KMT2D* as “tumor suppressors”, as there are multiple cases of significant rates *KMT2C* or *KMT2D* genetic duplication in tumors, which continue to be identified as copy-number analysis becomes more common. An investigation of breast cancer patient-derived-xenografts determined that *KMT2C* copy number rose significantly following serial transplantation suggesting a selective advantage of increased

expression [90]. Additionally, multiple studies have identified significant Kmt2d overexpression in tumors, correlating with proliferation, invasion, migration, and poor survival [91–93]. It is likely that many roles of MLR complexes are mechanistically involved in these malignancies.

Drosophila as a Model Organism

Drosophila melanogaster, also known as the fruit fly, has been used as a model organism for biological research for over a century [94–97]. Widely recognized for major breakthroughs in chromosomal inheritance and early advances in genetics, Drosophila has contributed to nearly every facet of eukaryotic research including development, gene regulation, metabolism, cell signaling, behavior, and a variety of human disease states. Although the fly has a genome roughly 5% the size of humans and other mammals, high conservation of gene sequences and cellular functions across metazoans has proven that discoveries within Drosophila biology are largely translational to other animals. Given this, the fruit fly is uniquely advantageous as a genetic and developmental model for both biological and technical reasons. Rapid generations, high proliferation, and genetic homogeneity ensure data collection volume that is unmatched against most other *in vivo* models. Additionally, the many decades of Drosophila research have yielded a fully sequenced genome and a plethora of tools to allow meticulous genetic alteration. Numerous critical biological questions cannot be addressed outside of living, developing organism. The use of Drosophila allows investigation that would not be as precise or statistically powerful otherwise.

The Gal4-UAS Expression System

One such tool widely used in Drosophila genetics in the Gal4-UAS expression system [98,99]. In short, this biochemical method allows modulation of the expression of individual gene

products in specific tissues and at specific developmental timepoints. This is achieved through the use of two transgenes, the first containing a Drosophila regulatory sequence controlling expression of the yeast transcription factor Gal4, and the second consisting of the Gal4-regulated upstream activating sequence (UAS) controlling expression of a gene product of the investigators' choice. Gal4 has no regulatory function on any endogenous Drosophila genomic targets and therefore when both genetic constructs are introduced into a Drosophila line (usually via mating) the Gal4 drives expression of the UAS transgene. Virtually any Drosophila regulatory sequence can be joined to regulate Gal4 transcription, allowing control of Gal4 expression in a consistent spatio-temporal manner; many such transgenes have been produced are collectively referred to as Gal4 drivers. A variety of gene products may be linked to the UAS sequence to allow for Gal4-controlled expression. These include endogenous Drosophila mRNAs to effectively overexpress or ectopically express proteins, exogenous gene mRNAs to introduce into the Drosophila background, mutated mRNAs to either investigate or take advantage of protein alteration, short hairpin siRNAs to knockdown expression of a specific mRNA through RNAi, or any other coding/non-coding regulatory transcript. I have taken full advantage of the robust flexibility of the Gal4-UAS system and each of these examples is used in experiments detailed in this dissertation.

The Drosophila Life Cycle

Drosophila is a holometabolous insect, meaning that its life cycle consists of multiple developmental stages including a period of metamorphosis that completely remodels its physiology [94]. At 25°C, complete development from embryo to adult occurs during approximately ten days. Approximately twenty-four hours after a female lays a fertilized

oocyte, the embryo develops and “hatches” into a first instar larva. There are three instar stages of larvae, each demonstrating an increase in size and each separated by a molt of their outer epidermal layer, or cuticle. The purpose of the larvae is to continuously consume nutrients, growing body mass and storing a large amount of energy as triglycerides (TAGs) in fat tissue. The first and second instar larva stages last approximately one day. The final larval stage, third instar, lasts approximately two days, during which time the animal rapidly grows in size. Once a sufficient body size and TAG storage level is reached, the animal abandons food consumption and begins to migrate vertically in order to begin pupariation. This final, largest larval phase is known as the wandering third instar larva. Pupariation involves the formation of a hard pupal case from the larval cuticle and begins the process of metamorphosis. Metamorphosis takes approximately five days and consists of multiple synchronous developmental processes as nearly every larval tissue is histolyzed and adult tissues are formed. Metamorphosis is completed as the fully-formed adult fly performs eclosion, the process of egress from the pupal case by inflation of the head tissue to break open the case and then forcing its way out. Following a brief period of adaptation a few hours after eclosion, adult flies are able to fly, eat, and mate, living for approximately ninety more days.

The development of adult organs from larval tissues during metamorphosis is a complicated process involving epithelial sacs known as imaginal discs [94]. An imaginal disc is simply the larval tissue that is destined to develop into adult-specific organ; each such organ has a corresponding disc or portion of a disc, including wings, eyes, antennae, mouth parts, legs, and genitals. Imaginal discs are excellent developmental model systems, as they consist of multipotent undifferentiated cells that systematically differentiate and develop into adult

tissues from the late larval stages continuing through metamorphosis. These discs can be isolated through dissection and inspected at many developmental time points, often in the wandering third instar larva.

Development of the Drosophila Compound Eye

The Drosophila eye disc (also called "eye-antennal disc") is the progenitor larval tissue that develops into the adult compound eye, antenna, and surrounding head capsule during metamorphosis [100–102]. Each larva harbors two identical eye discs, which independently form the left and right organs of the insect's head. Each disc is separated into two portions: the eye pouch and antennal section, which separately develop into the corresponding adult organs (**Fig. 2A**). The compound eye develops from the eye pouch in an unusual way which provides a unique opportunity for developmental research. While most imaginal discs mature into adult organs through synchronous differentiation across the tissue, the eye disc does so in an asynchronous wave. Differentiation into compound eye cells is first induced at the very posterior edge of the eye disc and then propagates to the anterior. This wave of differentiation is marked by a boundary called the morphogenetic furrow, caused by changes in cell morphology induced by differentiation signals. Therefore, all cells anterior to the morphogenetic furrow are undifferentiated eye cells, whereas all cells posterior are currently undergoing differentiation into compound eye units, or ommatidia. At the wandering third instar stage the furrow is normally approximately halfway through its progression across the eye pouch, resulting in equal sections of undifferentiated and differentiating eye tissue and allowing investigation of cells at different stages of organ development within a single contiguous tissue section.

The development of the 700-750 ommatidia of the adult compound eye is a meticulously controlled process which will not be discussed in complete detail here. It has been reviewed extensively before [103]. As an abridged overview, during and after the passage of the morphogenetic furrow cell fate is decided based on positioning of cells within emergent ommatidial eye fields. As these fields are established, an initial cell fate decision is between the proneuronal and interommatidial lineages; proneuronal cells at the center of each nascent ommatidium develop in photoreceptors , while interommatidial cells differentiate into pigment and bristle cells (**Fig. 2B**). After multiple stages of terminal differentiation and apoptotic pruning of excess cells, these will result in ommatidia each consisting of eight photoreceptors at the core capped by four cone cells at the distal margin and surrounded by pigment and bristle cells providing structure and insulation. The final result of this is a smooth and precisely patterned field of hexagonal ommatidial units, each bordering exactly six other ommatidia and each ommatidia bordered by exactly three bristles.

Numerous developmental signaling pathways are required for the accurate development of the compound eye from undifferentiated imaginal disc tissue. In the late second instar larva, Notch and EGFR signaling divides the disc into eye and antennal sections, respectively [104]. In the eye pouch Notch is active at the dorsal-ventral midline, driving expression of transcription factors such as Eyg that control eye fate and promote survival and proliferation of the undifferentiated eye cells [105]. Through a combination of Notch and EGFR activity in addition Hh signaling at the posterior of the eye pouch, differentiation is induced and the morphogenetic furrow begins to progress to the anterior [106]. The furrow and the wave of differentiation it represents is propagated by a positive feedback loop of Hh and Dpp signaling,

as a low Dpp gradient to the anterior of the furrow in undifferentiated tissue initializes cell cycle arrest and the commencement of differentiation [107]. Wg signaling at the dorsal and ventral margins of the developing compound eye suppress eye fate and allow for development of bordering bristles and head capsule cuticle [108].

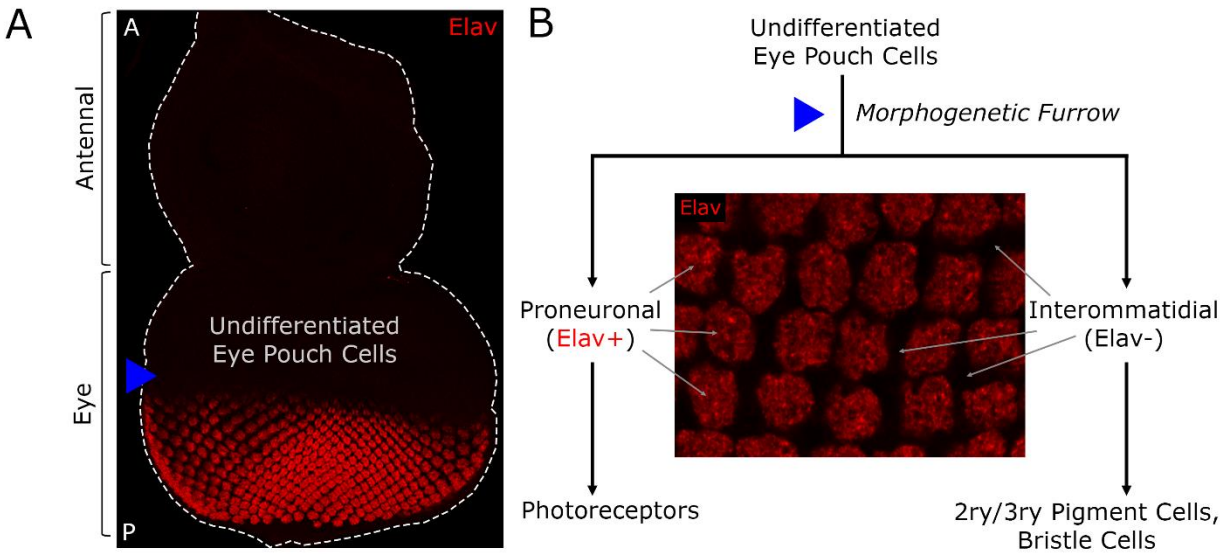

Figure 2. Attributes of the Drosophila Eye Disc. An eye disc is the progenitor tissue that develops into the adult eye, antenna, and surrounding head capsule during metamorphosis. **A.** The eye disc is separated into the anterior antennal portion and the posterior eye pouch. The eye pouch is further divided by the morphogenetic furrow (blue arrowhead), a mobile boundary inducing differentiation as it passes posterior to anterior. Therefore, eye tissue anterior to the furrow remains undifferentiated while that posterior to the furrow is differentiating into the compound eye units, known as ommatidia. Separate lineages of proneuronal (Elav+) and interommatidial (Elav-) cells can be visualized in these proto-ommatidia. **B.** A simplified schematic of cell fate choice during differentiation from undifferentiated eye disc cells in to proneuronal or interommatidial lineages.

The Drosophila *bantam* miRNA

Micro-RNAs (miRNAs) are small non-coding RNAs that function by regulating gene expression via inhibition of mRNA translation [109]. miRNAs are abundant within eukaryotes, and it is theorized that the use of these regulatory molecules evolved from the ancient RNA-

interference (RNAi) machinery developed as host defense against foreign nucleic acids from invading bacteria and viruses. Most miRNAs are spliced from long non-coding RNAs (lncRNAs) and undergo multiple rounds of processing while being loaded onto RNAi machinery and used as templates targeting specific mRNAs. These mRNAs contain targeting sequences, most often in the 3' untranslated region (3'UTR); translation of these transcripts is inhibited either through blockage of translation machinery and/or recruitment of endonucleases to cause cleavage and degradation. A single miRNA tends to have many different target mRNAs, and the target sequence is often an inexact pair match, causing lower affinity binding and bulging of the double-strand with functional consequence. Similar to enhancers, the expression of miRNAs is a precisely controlled regulatory network that is necessary during eukaryotic development; this critical activity of this post-transcriptional regulatory system is still being fully elucidated.

The Drosophila *bantam* miRNA is generated from a ~12kb non-coding precursor RNA (CR43334); the *bantam* locus spans nearly 40kb including multiple tissue-specific enhancers responsible for regulating proper expression levels [110,111]. *bantam* is transcribed during development to regulate cellular survival, proliferation, migration, as well as organ growth and patterning via translation inhibition of multiple target mRNAs including actin regulator *Ena*, cell cycle regulator *Trbl*, Myc regulator *Mei-P26*, splicing regulator *Rox8*, hormone biosynthesis enzyme *Jhamt*, as well as neural stem cell factors *Brat* and *Pros*. [112–119]. The best-characterized role of *bantam* is translation inhibition of the proapoptotic transcript *hid* during organ development [112]; overexpression of *bantam* in the differentiating eye disc tissue posterior to the morphogenetic furrow both suppresses the deleterious effects of *hid* overexpression and causes ommatidial malformation by suppressing developmental apoptotic pruning [112,120].

To regulate the critical functions of *bantam*, expression of the miRNA is controlled by various developmental signaling pathways. The Hippo signaling effector Yorkie (Yki) transcription factor positively regulates *bantam* transcription during organ growth and binds to multiple *bantam* regulatory regions with heterodimeric partners Scalloped (Sd) or Homothorax (Hth), including tissue-specific enhancers [110,111,120,121]. Yki also partners with TGF-β/Dpp effector Mad to promote *bantam* expression [122]; separate from Hippo signaling, Dpp signaling positively regulates *bantam* through multiple direct and indirect methods including downregulation of repressor Brinker (Brk) and upregulation of activator Omb [123–125]. Notch signaling activity has been shown to either promote or repress *bantam* expression based on developmental context [113,126]. In addition to being regulated by Hippo, Dpp, and Notch signaling, *bantam* miRNA acts as a feedback inhibitor of these pathways through translation inhibition of SdBP, Mad, and Numb, respectively [125–127]. Appropriate *bantam* expression regulation by and subsequent feedback regulation of these developmental pathways is required for proper organismal development.

The Drosophila Fat Body

The Drosophila fat body is a homogenous organ that varies in structure and size depending on developmental stage [91]. Orthologous to mammalian adipose and hepatic tissue, its functions include but are not limited to: the synthesis, maintenance, and mobilization of TAGs for energy storage [128,129]; regulation of organismal metabolism in response to feeding or starvation [130–132]; and systemic stress response, including production of antimicrobial peptides and other stress response proteins [133–135].

During Drosophila embryogenesis, certain cells of the mesoderm adopt a pre-adipocyte fate, coalescing into a sheath of tissue that develops into the larval fat body [136]. This organ

extends throughout the body length of the larva, enveloping other organs and systems and in constant direct contact with the hemolymph, the interstitial transportational fluid of the animal [137]. Through larval development and the increasing instar stages, the fat body enlarges considerably and makes up a significant portion of body mass in the wandering third instar. However, this growth is not due to cell division but rather endoreplicative cellular expansion as TAGs are stored in lipid droplets [137,138]. During metamorphosis, the larval body is heavily restructured and fragmented by autophagy and apoptosis, transforming from contiguous tissue into small clumps of cells that migrate throughout the developing adult, settling mainly under the abdominal epidermis but also within the head capsule [137,139].

The Fat Body as a Triglyceride Reservoir

After feeding, the Drosophila digestive tract catabolizes carbohydrates, lipids, and proteins into metabolites that are released into the hemolymph and converted into molecular nutrients for the animal's tissues. During these periods of nutrient availability, the fat body uptakes these metabolites and converts them into forms of stored energy: glycogen and TAGs. TAGs, stored in large cytosolic lipid droplets, are the main source of metabolic energy used during non-feeding periods, including starvation due to lack of food as well as metamorphosis. This is accomplished through lipases such as Bmm or Lip3, which convert TAGs into diacylglycerol for loading onto lipoprotein particles that are shuttled into the hemolymph for systemic delivery [128]. The storage and depletion of TAGs in fat body cells are regulated by antagonistic signaling pathways sensitive to feeding and stress states [140]. During periods of feeding, insulin-like peptides (Ilps) promote storage and inhibit depletion of fat body TAGs [141]. Fat storage is suppressed and depletion promoted by multiple signals, including adipokinetic

hormone (Akh) during starvation [142], ecdysone (Ec) during developmental transformation [143,144]. These signals induce transcriptional changes through directly activating membrane or nuclear receptors as well as indirectly affecting activity of other transcription factors, including the master stress response factor Foxo [129,145–147].

The Foxo Transcription Factor and Stress Response Signaling

When confronted by stressful conditions, healthy metazoan cells must respond by altering their homeostasis in order to endure and combat the toxic situations. This is accomplished though both post-translational alterations and transcriptional reprogramming. These stress response genes are poised in a state of paused transcription initiation, awaiting release by contact with activated transcription factors and distal regulatory elements [148,149]. While many reactive changes in gene expression are specific to the type of cellular stress face, many are common among the various stresses; these include cell cycle regulators, stabilizing chaperone proteins, antioxidant enzymes, metabolic regulators, autophagy and apoptosis machinery. These targets are all regulated by the Foxo family of transcription factors [150–152]. Thus, Foxo factors are used as master regulators of cellular stress, the fulcrum of the axis balancing healthy homeostasis and stress response. While larger metazoans contain multiple Foxo paralogs that vary in tissue specific expression and activity, Drosophila contain a single Foxo that regulates stress response in all cells [153,154].

Foxo is regulated by post-translational modification controlling cellular localization. Under growth conditions and in the absence of stress, Foxo is phosphorylated by PI3K effector kinase Akt (feeding signals) and MAPK effector kinase Erk (growth signals), allowing cytosolic sequestration by 14-3-3 proteins [150]. Under stress conditions MAPK effector kinase Jnk (stress

signals) phosphorylates Foxo at other residues, promoting nuclear localization and transcriptional activity [155]. The presence or absence of these various signals converging on Foxo allow it to act as a rheostat of the general stress level of the cell, regulating stress response transcription according to severity.

Aging processes in many metazoan species are sensitive to the activity of Foxo proteins, likely through their role in regulating cellular metabolic and stress states [156]. Overexpression of Foxo in the Drosophila fat body significantly enhances longevity, suggesting that Foxo activity in this organ is particularly critical for responding to periods of stress [157]. During starvation, Foxo activity is promoted throughout the organism primarily due to loss of repressive Akt activity. Within cells of the fat body, this results in adipocyte-specific changes in gene expression in addition to the normal suite of Foxo-driven stress response genes.

Foxo is Required for Ilp6 Expression in the Fat Body

Drosophila growth in response to feeding and hormone signals is mediated through eight insulin-like peptides (Ilps), endocrine signaling molecules that regulate a network of processes governing systemic metabolism, stress response, and development [158]. While most of these Ilps are secreted from insulin-producing cells (IPCs) of the central nervous system, Ilp6 is produced primarily from the fat body. Ilp6 is expressed during metamorphosis as well as starvation, and is responsible for regulating growth during periods of non-feeding [132]. Foxo activity is necessary for Ilp6 expression during these periods, and it is through Ilp6 that Foxo mediates its ability to prolong longevity, likely through crosstalk between the fat body and IPCs, reprogramming organismal metabolism [132,159].

Relish and Foxo Regulate Expression of Antimicrobial Peptides

While not all metazoans possess an adaptive immune system able to specifically target previously-encountered pathogens, ancient innate immune processes are highly conserved among species. Among these processes is the expression and secretion of antimicrobial peptides (AMPs), which damage invasive bacteria and fungal cells without deleteriously affecting native tissue [160]. While epidermal cells of the Drosophila cuticle and digestive tract are able to express these peptides, the major source for circulating AMPs is the fat body [161]. Conventionally, AMP expression is induced by the activities of the Toll or Imd pathways. These pathways sense invasive microbial motifs and lead to activation of the NF-κB transcription factors Dl, Dif, or Relish, which drive AMP transcription [162,163]. However, AMPs are also expressed independently of Toll/Imd signaling in response to developmental changes, starvation, or other forms of stress [164]. Foxo is responsible for directly regulating fat body AMP transcription in these situations, and this expression indirectly impacts longevity and aging processes of the animal through unknown mechanisms [164,165].

Foxo Regulates Expression of Lipase Brummer

Brummer (Bmm) is a transcriptional target of Foxo in the fat body [166]. Bmm is an evolutionarily conserved triglyceride lipase (orthologous to human adipocyte triglyceride lipase ATGL) responsible for the rate-limiting step of mobilizing TAGs stored in lipid droplets and processing them into diacylglycerol for further fatty acid metabolism or shuttling into circulation [167]. As a key regulator of TAG lipolysis, Bmm is primarily transcriptionally regulated: downregulated during times of TAG storage and feeding and upregulated during nutrient stress. Foxo activity is used as a primary method for controlling Bmm expression and modulating the

fat body's lipolytic response to starvation, ensuring that the TAG depletion rate allows for survival without excessive exhaustion of energy stores [159,166,168]. As the precise control of lipolysis during nutrient stress is critical for survival, this regulatory activity is precisely modulated by parallel mechanisms. During starvation, Imd pathway effector Relish acts antagonistically to Foxo, suppressing Bmm expression to prevent excess TAG depletion [168].

Dissertation Objectives

As mutation of human *KMT2C* and *KMT2D* are associated with oncogenesis as well as developmental syndromes, alteration of MLR complex activity is clearly foundational to disease states. However, the mechanisms leading from MLR subunit mutation to disorder are not yet understood. Previous *in vitro* research using mammalian cell culture has identified dysregulated pathways and transcriptional targets, yet *in vivo* analysis is required to properly characterize the consequences of altered MLR activity in developing tissue. Due to the high conservation of MLR complex activity among animals, characteristics of MLR complexes in model organisms will likely translate to humans. My objective is to use to fruit fly *Drosophila melanogaster* as a genetic model to further elucidate the functions of MLR complexes during development as well as stress states.

Determination of Mechanisms Requiring MLR Complex During Eye Development

A previous report by our group identified rough and shrunken compound eye phenotypes resulting from knockdown of *Cmi* or *trr* expression within the eye disc. These results suggest alteration of cell survival and/or developmental signaling pathways during organ formation. I use the compound eye as a developmental model to further characterize the regulatory targets and functions of the MLR complex.

Characterization of MLR Complex Regulation of Fat Body Development and Function

Tissue-specific murine loss of function studies have determined that MLR complex activity is required for adipose tissue development as well as bile acid homeostatic regulation by hepatic tissue. The Drosophila fat body is orthologous to both mammalian fat and liver tissue. I alter MLR complex activity in the fat body via modulation of Cmi level in order to identify regulatory targets and functions of the MLR complex.

CHAPTER 3

MATERIALS AND METHODS

Drosophila Culture and Husbandry

All stocks were maintained at 25°C on standard Drosophila cornmeal-yeast-dextrose medium (6% cornmeal, 3% yeast extract, 13% dextrose, 1% agar, 0.25% methylparaben antifungal) unless otherwise indicated. OregonR used as wild type strain. All other fly strains and transgenic lines obtained from Bloomington Drosophila Stock Center (BDSC) unless otherwise indicated (**Table 1**). All fly strains described in Flybase (http://flybase.bio.indiana.edu). Mating crosses and/or animal development maintained at 25°C on standard medium unless otherwise indicated.

Table 1. Fly Stocks Used

Genotype	Source
OregonR (Wild Type)	(Chauhan 2012)
Dcr2(X);UAS-Cmi-IR(III)	(Chauhan 2012)
UAS-Cmi	(Chauhan 2012)
UAS-Trr-IR	BDSC #29563
Ey-Gal4	BDSC #8220
GawB69B	BDSC #1774
DE-Gal4/TM6B	BDSC #78371
GMR-Gal4	BDSC #1104
C765-Gal4	BDSC #36523
En-Gal4	BDSC #30564
UAS-bantam	BDSC #60671
banGFP-sensor (Tub-EGFP.ban)	Dr. Richard Mann
bee-LacZ (51D)	Dr. Richard Mann
bwe-LacZ (86Fa)	Dr. Richard Mann
UAS-bansponge	Dr. Stephen M. Cohen
UAS-p35	BDSC #5072
N^{spl-1} (hypomorphic mutant)	BDSC #118
UAS-Numb	BDSC #51663
UAS-Numb-IR	BDSC #35045
UAS-Eyg	BDSC #26809
NRE-GFP (II)	BDSC #30727
Lsp2-Gal4	BDSC #6357
UAS-Foxo	BDSC #80946
UAS-Foxo-IR	BDSC #32993

Gal4 Expression System

Tissue- and temporal-specific expression of inverted repeats or overexpression constructs was accomplished using the Gal4-UAS transgenic expression system [98,99]. Two transgenes, the first containing tissue-specific Drosophila regulatory sequences controlling expression of yeast transcription factor Gal4, and the second containing Gal4-activated UAS regulatory sequences controlling expression of the desired gene product, were introduced in the same animal through mating. This process drives expression on the desired gene product according the activity of the Drosophila regulatory sequences used. (Gal4 drivers and UAS target transgenes listed in **Table 1**).

Generation of Recombinants

To generate the Ey-Gal4,bee-51D recombinant second chromosome, virgin Ey-Gal4 females were mated with bee-51D males. From the heterozygous offspring, virgin females harboring potential gametic recombination events were mated with wg^{sp-1}/CyO males in order to protect any potential recombinant chromosomes over the balancer CyO chromosome. Resulting males were individually pair-mated with wg^{sp-1}/CyO virgin females, and once offspring larvae were observed the males were collected and homogenized for PCR (see next section on Polymerase Chain Reaction). Progeny of a male positive for both Gal4 (Ey-Gal4) and LacZ (bee-51D) genes were mated with each other to form a homozygous line containing the recombinant Ey-Gal4,bee-51D chromosome.

To generate the Cmi-IR,Lsp2-Gal4 recombinant third chromosome, virgin Cmi-IR females were mated with Lsp2-Gal4 males. From the heterozygous offspring, virgin females harboring potential gametic recombination events were mated with w-;TM3/TM6B (WTT) males in order

to protect any potential recombinant chromosomes over a balancer chromosome. Resulting males were selected by eye color (darker eyes suggesting presence of both transgenes) and individually pair-mated with WTT virgin females. Once offspring larvae were observed the males were collected and homogenized for PCR (see next section on Polymerase Chain Reaction). Progeny of a male positive for Gal4 (Lsp2-Gal4) were also tested for presence of Cmi-IR by mating with Ey-Gal4 flies and checking for presence of the rough and shrunken eye phenotype in the offspring. Positively verified lines were mated with each other to form homozygous lines containing the recombinant Cmi-IR,Lsp2-Gal4 chromosome.

Polymerase Chain Reaction

For single animal polymerase chain reaction (PCR), a single adult was homogenized in 20 µl of Fly Grinding Buffer (10mM Tris-HCl pH7.8, 1mM EDTA-Na-2H_2O, 25mM NaCl, and 0.2 mg/ml freshly-thawed proteinase K) in a microcentrifuge tube using a plastic pestle. Using a BioRad C1000 Touch™ Thermal Cycler, homogenates were heated at 35°C for 30 minutes to promote enzymatic activity and then heated at 95°C for 5 minutes to kill enzymatic activity. Homogenates were kept at 4°C overnight or frozen for future PCR verification.

For PCR verification, 1uL of homogenate was added to 12.5uL DreamTaq Green Master Mix (Thermo), 2.4µL forward primer of choice, 2.4µL reverse primer of choice, and 6.7µL ddH_2O in a PCR tube. Tubes were vortexed to mix and exposed to PCR protocol on a BioRad C1000 Touch™ Thermal Cycler. PCR protocol was initiated by heating at 95°C for 5 minutes to melt genomic DNA. Then the following cycle was repeated 30 times: melting at 95°C for 30 seconds, annealing at the desired temperature (see **Table 2**) for 30 seconds, and extension at 72°C for 30 seconds. After all cycles were completed the samples were exposed to a final extension step at

72°C for 5 minutes. PCR products were kept at 4°C overnight or frozen for future gel electrophoresis.

For gel electrophoresis, an agarose gel was created by boiling 2% agarose in TBE buffer (45mM Tris base, 45mM boric acid, 2nM EDTA (from pH8.0 solution)), then adding 0.5ug/mL ethidium bromide and mixing, and finally transferring to a mold for polymerization. TBE was added to submerge polymerized gel and 20uL each of the PCR products were run through the gel at 100V voltage. Gels were visualized on High Performance Ultraviolet Transilluminator (UVP).

Table 2. PCR Conditions

Target	Forward Sequence	Reverse Sequence	Annealing Temp.	Extension Time	Cycles	Amplicon Length
Gal4	CCGAATTTGGTGGTCTGTCT	AATTGGTTAGAGCGGTGGTG	55°C	30 seconds	30	330 bp
LacZ	ACTATCCCGACCGCCTTACT	TAGCGGCTGATGTTGAACTG	52°C	30 seconds	30	200 bp

Phenotypic Scoring

Animals of the desired genotype developed at 29°C for efficient Gal4 activity. Resulting adult Drosophila were scored for eye or wing phenotypes individually while anesthetized by CO_2 exposure under a dissecting microscope at 10-63X magnification. Total number of individuals analyzed displayed as N values in corresponding figures.

For analysis of rough and shrunken eye phenotypes, individual animals were binned into categories according to closest appraised phenotype: “wild type” if eye size approximates wild type size with no apparent roughness; “slightly rough and shrunken” if eye ~70-80% of wild type size and <50% of eye surface demonstrates roughness; “completely rough and shrunken” if eye ~20-60% of wild type size and >50% of eye surface demonstrates roughness; “malformed organs” if eye ~<20% of wild type eye size or if entire eye and/or antennal organs are missing or

duplicated. Significant difference of eye phenotype severity between genetic populations was measured using Pearson's Chi-Squared Test.

For analysis of wing vein phenotypes, individual wings were scored according to retraction or splitting of individual veins as well as presence of ectopic veins. General wing size was also noted if clearly different than wild type.

Tissue Preparation, Immunostaining, and Fluorescence Microscopy

Eye or wing imaginal discs were dissected from wandering third instar larvae in ice-cold PBS and transferred to 4% formaldehyde in PBS for 15-20 minutes. Tissues were then washed three times in PBST (PBS + 0.1% Triton-X100) before transferred to a blocking solution of PBSTB (PBST + 0.1% Fetal Bovine Serum) for at least 2 hours. Afterwards tissues were incubated in primary antibody solution at 4°C overnight. Tissues were then washed twice in PBSTB for five minutes each, once in PBSTB + 2% NGS (Normal Goat Serum) for 30 minutes, and then twice in PBSTB for 15 minutes each. Afterwards tissues were incubated in secondary antibody solution in the dark at room temperature for 2 hours. Tissues were washed three times in PBST for 5 minutes each before being mounted in ProLong Gold antifade reagent with DAPI (Invitrogen) on a glass slide for imaging. The experimental and control samples were stained in parallel in all cases.

Primary antibodies included mouse α-β-Gal (JIE7) and mouse α-Elav (9F8A9) (Developmental Studies Hybridoma Bank/Univ. of Iowa), rabbit α-GFP (GenScript) and rabbit α-Dcp-1 (Asp216) (Cell Signaling Technologies). Guinea pig α-Cmi was generated as previously described [11]. Primary antibodies were used at 1:1000 concentration, except α-Dcp-1 was used at 1:250 concentration. Secondary antibodies were used at 1:1000 concentration and included

α-Mouse, α-Rabbit, and α-Guinea Pig IgG (H+L) conjugated to Alexafluor 488 or 568 fluorophores (Life Technologies).

Compound microscopy images were captured using an Olympus BX53 microscope with a Hamamatsu ORCA Flash 4.0 LT camera. Confocal microscopy images were captured using a Zeiss LSM 880 Airyscan and processed using Zeiss Zen® software.

TUNEL Staining

TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining accomplished by dissecting, fixing, and washing imaginal discs a described in section 3.4 before following manufacturer's protocol using the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics). In short, fixed tissues were incubated in TUNEL solution (90% fluorescein-dUTP label solution, 10% TdT enzyme solution) for 90 minutes at 37°C in a dark humidity chamber, then washed three times in PBST for 5 minutes each and mounted for imaging.

***bantam* Sensor**

The *bantam* sensor is a transgene encoding enhanced green fluorescent protein EGFP containing two perfect 31bp *bantam* target sequences in the 3'UTR regulated by a constitutively-active αTub84B promoter fragment [112] (**Fig. 3**). Therefore, GFP expression acts as an inverse reporter of *bantam* activity, a readout of *bantam* expression. *bantam* sensor activity was assayed through staining for GFP expression (section 3.4).

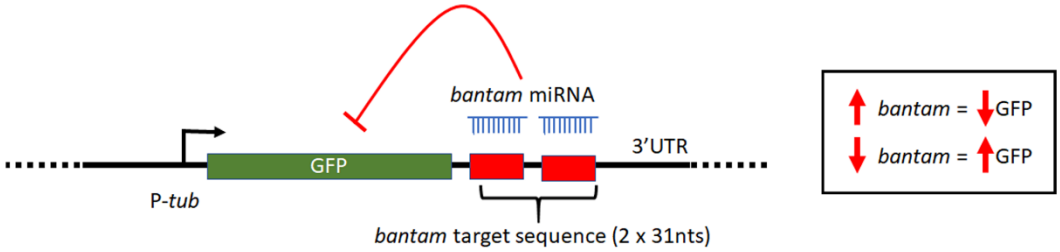

Figure 3. The *bantam* Sensor. The *bantam* sensor inverse reporter construct (*ban*sensGFP) in composed of a constitutively expressed EGFP transcript with multiple *bantam* miRNA target sites in the 3' UTR. Therefore, the higher the levels of *bantam* the greater the translation inhibition of the transcript leading to lower GFP expression, and visa-versa.

Adult Wing Dissection and Mounting

Wings were dissected from adult animals and dehydrated in isopropyl alcohol for 20 minutes. After dehydration, wings were mounted in DPX mountant (Fluka) on glass slides. Images were captured using a Leica MZ16 microscope with Leica DFC480 camera.

Fluorescence Intensity Quantification

Quantification of signal mean fluorescence intensity in eye or wing discs was assayed using Fiji ImageJ software to measure fluorescence intensity as mean grey value of selected areas, subtracting background (signal-negative sections of imaginal disc tissue used as background) (Fiji: [169]). Significant difference of mean fluorescence intensity in eye and wing discs was measured using Student's T-test.

Scanning Electron Microscopy

Adult eyes were prepared in parallel for scanning electron microscopy (SEM) using critical point drying as previously described [170]. SEM photography was taken at 1500X magnification using a Hitachi SU3500 microscope.

Collection of Fat Body Tissue for RNA-seq

Wandering third instar larvae were collected and dissected in ice cold PBS. Entire fat body tissue from twenty-five larvae was extracted, ensuring no collection of other tissue types, and placed in ice cold PBS. Each collection was flash frozen with dry ice until preparation of tissue for RNA extraction.

Metamorphosis Survival

Wandering third instar larvae were placed in vials and allowed to undergo metamorphosis at either 25°C, 29°C, or shifted between the two temperatures at a midpoint of metamorphosis. For temperature shift experiments, tubes containing pupae were transferred from the initial to the secondary temperature at approximately 50 hours post-pupariation (~stage P7 according to Bainbridge and Bownes pupal staging) [171].

Survival was recorded by tallying number of living newly-eclosed adults per number of larvae placed in each vial. “Pharate Lethal” was defined as a fully formed pharate in the pupal case that is not moving or beginning eclosion (stage P14 according to Bainbridge and Bownes pupal staging) and is by all appearances dead, not responsive to stimuli or to removal from pupal case. The experimental and control animals were staged and placed in parallel in all cases. “Eclosion Lethal” was defined as a fully-formed adult in the process of eclosion from the pupal case (stage P15(ii) according to Bainbridge and Bownes pupal staging) and is by appearances dead, not responsive to stimuli or to removal from pupal case.

Adult Starvation Survival and Refeeding

Newly-eclosed adults (0-5 hours post-eclosion) were transferred to vials containing starvation media (2% agar and 0.25% methylparaben antifungal) to provide moisture but no

carbohydrates, amino acids, lipids, or other nutrients. Survival was assayed by tallying clearly living flies (assayed by movement) at regular timepoints until death of all individuals. The experimental and control individuals were starved and assayed in parallel in all cases. Results were plotted as survival probability according to Kaplan-Meier estimation and statistical difference between control and experimental populations was measured using the log-rank test.

For aged starvation, newly-eclosed adults were separated according to sex and kept on standard media for 4 days to allow for TAG levels to reach adult homeostasis. Animals were then transferred in parallel to starvation media and survival was assayed similarly.

For refeeding assay, newly-eclosed adults were transferred to starvation media for 24 hours, at which point they were transferred to standard medium. For use in TAG quantification, three samples of each genotype were collected immediately after eclosion, after 1 day starvation, after 1 day refeeding, and after 2 days refeeding. Each replicate consisted of five animals homogenized.

Oxidative Stress Survival

Oxidative stress media was prepared by adding to the standard food recipe hydrogen peroxide (H_2O_2) to final concentration of 5%.

Newly-eclosed adults were separated by sex and aged for three days before being placed on starvation media for 4 hours in induce feeding behavior. They were then transferred to the oxidative stress media and assayed for survival regularly until complete lethality.

Triglyceride Quantification

For each replicate, 5 animals were washed in cold PBS before being homogenized in 500µL PBS + 0.05% Tween20 (PBST) in a microcentrifuge tube using a plastic pestle. 20µL of homogenate was set aside for Bradford protein assay. Remaining homogenate was heated for 10 minutes at 70°C to kill enzymatic activity. 20µL of heated homogenate each was set aside into two microcentrifuge tubes: a reagent tube and a background tube. To the reagent tube, 20µL of Triglyceride Reagent (Sigma) was added to cleave hydrocarbon tails from glycerol. To the background tube, 20µL of PBST was added. All tubes were incubated for 60 minutes at 37°C to promote enzymatic activity. Afterwards tubes were centrifuged for 3 minutes at 10,000 rpm. A standard curve ranging from 0.0625-1.0 mg/mL glycerol standard (Sigma) was diluted from stock into PBST. (The standard curve included points at 0.0625, 0.125, 0.25, 0.5, and 1.0 mg/mL.) 30uL of each standard (including blank) and sample was transferred to a 96-well plate. For colorimetric assay, 100uL Free Glycerol Reagent (Sigma) was added to each well and the plate was incubated at 37°C for 5 minutes to promote enzymatic activity. Absorbance was measured at 540nm on a BMG Labtech POLARstar® Omega plate reader.

For Bradford protein assay, a standard curve ranging from 0.094-1.5 mg/mL bovine serum albumin (BSA) was diluted from stock into ddH_2O. (The standard curve included points at 0.09375, 0.1875, 0.375, 0.75, and 1.5 mg/mL.) 10µL of each standard (including blank) was transferred to a 96-well plate. For each homogenized sample, 10µL was transferred to one well and 5µL was diluted in 5µL ddH_2O in a second well to test linearity and ensure fit on standard curve. 300µL “Coomassie Plus – The Better Bradford Assay” reagent (Thermo) added to each

well and the plate was allowed to sit at room temperature for 10 minutes. Absorbance was measured at 595nm on a BMG Labtech POLARstar® Omega plate reader.

TAG concentration for each replicate was calculated by calculating the concentration of each sample well using the standard curve, and then subtracting the background tube concentration from the reagent tube concentration. Protein concentration for each replicate was calculated by calculating the concentration of each sample well using the standard curve. Average animal TAG levels were calculated by dividing TAG concentration by protein concentration for each replicate and then dividing by 5 (for each animal per replicate).

CHAPTER 4

RESULTS

Knockdown of Cmi or Trr in the Developing Eye Causes Rough and Shrunken Organs

It has been previously demonstrated that the MLR COMPASS-like complex is necessary in the developing compound eye for proper organ formation and patterning. The classical shrunken eye phenotype Bar^{1} is enhanced in a genetic background heterozygous for *Trr* null mutant Trr^{1}, likely due to reduced Hedgehog-Dpp signaling [51]. Identification and characterization of *Cmi* revealed that knockdown of the gene through eye-specific expression of a Cmi-specific RNAi hairpin results in reduced eye size as well as occasional loss of antennal structures [11]. To further validate and characterize these effects, I expressed either that RNAi hairpin containing a *Cmi*-specific inverted repeat to drive *Cmi* knockdown (Cmi KD) or a hairpin containing a *Trr*-specific inverted repeat to drive *Trr* knockdown (Trr KD) under the control of the Eyeless-Gal4 driver (Ey-Gal4), which drives expression within the entire eye pouch. These resulted in a rough and shrunken eye phenotype varying in penetrance and expressivity (**Fig. 4A**). The Cmi KD phenotype demonstrated approximately 75% penetrance with ~25% of the population displaying wild type eyes. The affected individuals were categorized as exhibiting either “slightly” or “completely” rough and shrunken eyes, with a small percentage displaying severely reduced eye tissue and/or missing or duplicated antennae characterized as “severely malformed”. Trr KD resulted in a more severely affected population displaying complete

penetrance of the phenotype with all individuals scored as “completely rough and shrunken” or “severely malformed”. Roughness of compound eyes suggest a disruption in ommatidial patterning. Therefore, scanning electron microscopy (SEM) was used to analyze the ommatidial structures in the Cmi KD and Trr KD populations. The consistent hexagonal patterning of the compound eye is disrupted on knockdown of either Cmi or Trr (**Fig. 4B**). In the wild type eye, each ommatidial unit is covered by a lens and is bordered by six neighboring units sharing equal sides. Every other corner of the hexagonal lens is in contact with a single bristle, meaning that each ommatidia is bordered by three bristles and each bristle is surrounded by three ommatidia. In both Cmi KD and Trr KD eyes, a variety of sporadic effects disturb this pattern: bristles are absent or duplicated, bristles occur at incorrect ommatidial junctions, neighboring lenses are fused, and ommatidial crowding alters number of bordering ommatidia as well as the length of those borders. Just as seen within the population data, Trr KD appears to cause more frequent and severe ommatidial defects than Cmi KD (**Fig. 4B**).

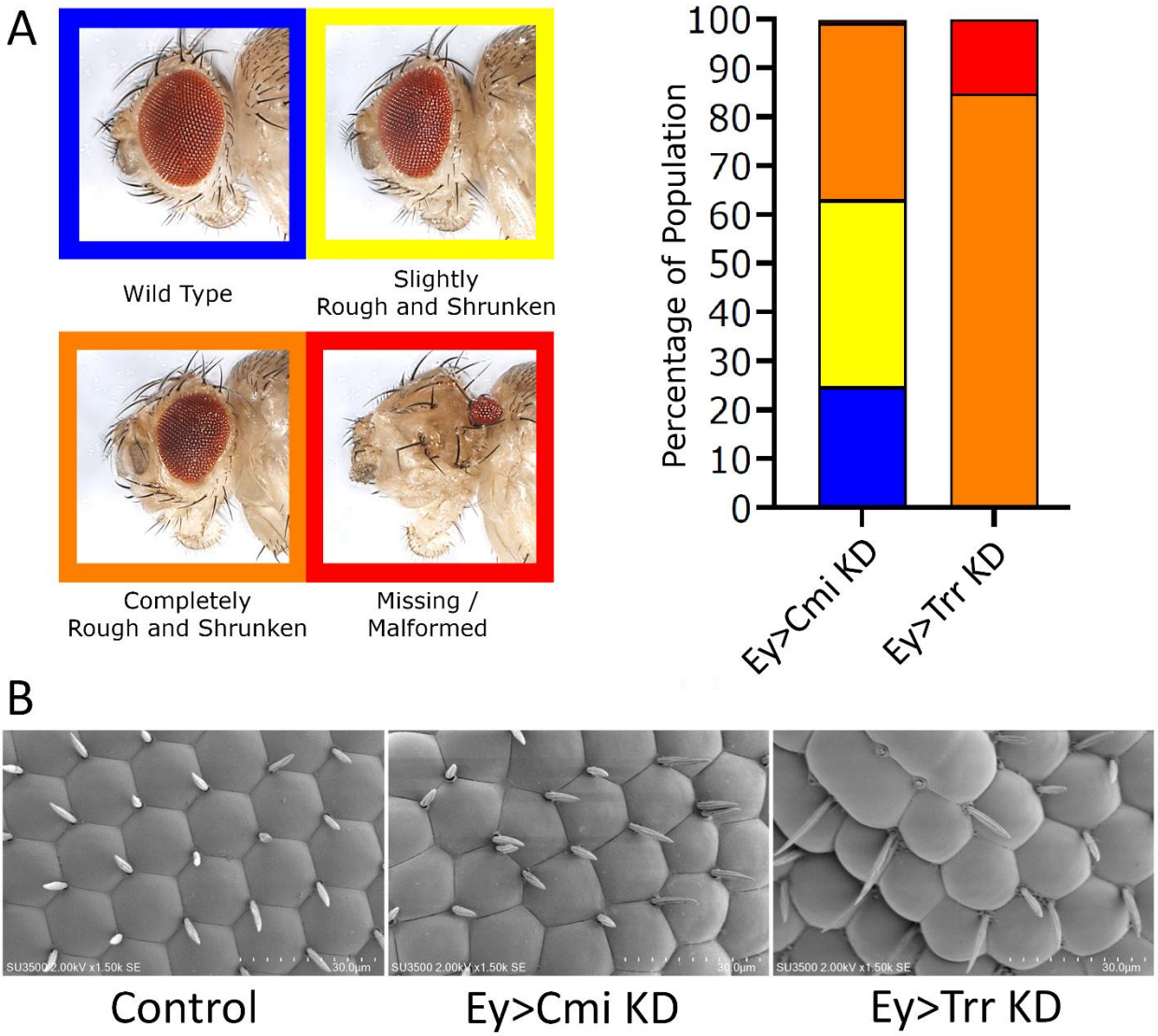

Figure 4. Knockdown of *Cmi* or *trr* in the Eye Disc Causes a Rough and Shrunken Adult Eye. *Cmi* or *trr* was knocked down (KD) in the entire eye pouch via expression of RNAi constructs driven by Ey-Gal4, causing a rough and shrunken phenotype with varied levels of penetrance and expressivity. OregonR strain used as wild type control. **A.** The Cmi knockdown phenotype is ~75% penetrant with relatively low expressivity compared to the Trr KD phenotype, which is completely penetrant with high expressivity. (*N > 300 for each genotype*) **B.** As seen via SEM, the roughness is due to ommatidial mispatterning of the compound eye, including fused lenses, altered ordering, and missing/duplicated bristles.

The MLR Complex Promotes Cell Survival in Undifferentiated Imaginal Tissue

The reduced organ size and altered cellular patterning caused by *Cmi*/*Trr* depletion in the eye disc suggests aberrant cell death during development: dysregulated apoptotic pruning of cells during compound eye formation leads to patterning defects and "roughness" of the eye surface [173,174]. Additionally, loss of developing eye tissue may cause neighboring cells to proliferate and fill the lost area through a process known as compensatory proliferation [172]. While compensatory proliferation during development is normally effective at seamless replacement of dead cells for proper organ formation, if the neighboring cells are of a different fate than the lost cells then organ size and tissue patterning becomes disrupted. Therefore, I investigated potential alteration of cell survival in *Cmi*/*Trr* knockdown eye discs. Staining for the cleaved form of effector caspase Dcp-1 demonstrated a clear increase in cells positive for active caspase in knockdown discs compared to control (**Fig. 5A**). This effect was observed only within the undifferentiated eye tissue anterior to the morphogenetic furrow and, intriguingly, was concentrated on the dorsal-ventral midline of the disc. In order to verify that these data represented cells undergoing caspase cascade leading to apoptosis, the classic apoptotic assay of TUNEL staining was used (**Fig. 5B**). TUNEL results were identical to Dcp-1 staining, demonstrating that upon depletion of MLR subunits, undifferentiated eye cells on the dorsal-ventral midline induce apoptotic cell death. As *Cmi*/*Trr* are knocked down throughout the entire eye pouch, this effect may either be cell-autonomous (caused by reduced MLR activity in the dying cells themselves) or non-autonomous (caused by reduced MLR activity in other cells dysregulating signals to the dying cells). To test this, *Cmi*/*Trr* were knocked down only within the dorsal half of the eye pouch, leaving the ventral half an internal control expressing wild-

type levels (**Fig. 5C**). Only the cells expressing the knockdown constructs displayed the Dcp-1 activation, demonstrating that the effect is intrinsic to cells with reduced MLR activity. This suggests that dysregulated expression of one or more transcriptional targets of the MLR complex induces apoptosis in undifferentiated eye cells.

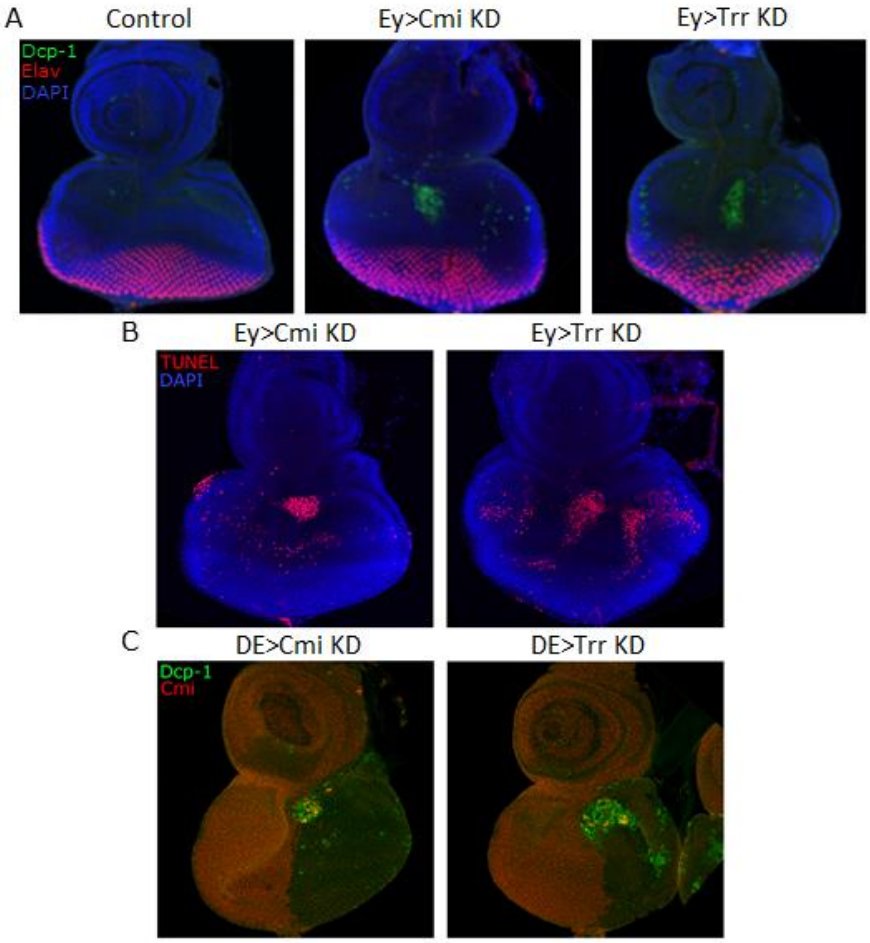

Figure 5. Knockdown of *Cmi* or *trr* in the Eye Disc Causes Increased Apoptosis in Undifferentiated Cells. A. Eye discs from W3L were stained for Dcp-1 (green) to marked activated effector caspase and Elav (red) to distinguish differentiating cells. Cmi or Trr KD lead to increased caspase activation solely within undifferentiated eye tissue, with a significant concentration on the dorsal-ventral boundary. *OregonR/En-Gal4* genotype used as control. **B.** TUNEL staining (red) was used as a second marker of apoptotic cell death, confirming the interpretation of Dcp-1 staining. **C.** DE-Gal4 was used to test cell autonomy of the apoptotic effect. Eye discs were stained for Dcp-1 (green) and Cmi (red) to distinguish cells experiencing KD. Caspase activation occurs solely within cells knocking down Cmi or Trr and not in neighboring tissue; the effect is cell autonomous.

To investigate if this apparent protective role of the MLR complex is eye-specific or conserved in other imaginal tissues, I used the En-Gal4 driver to knockdown *Cmi* or *Trr* in the posterior margin of the wing disc (**Fig. 6**). While this did not result in a single concentrated area of caspase activation, as in the eye, depletion of the MLR subunits did cause a general increase in caspase activation in knockdown tissue compared to neighboring control tissue. These results suggest that the MLR complex plays a role in suppressing apoptosis in undifferentiated imaginal tissue.

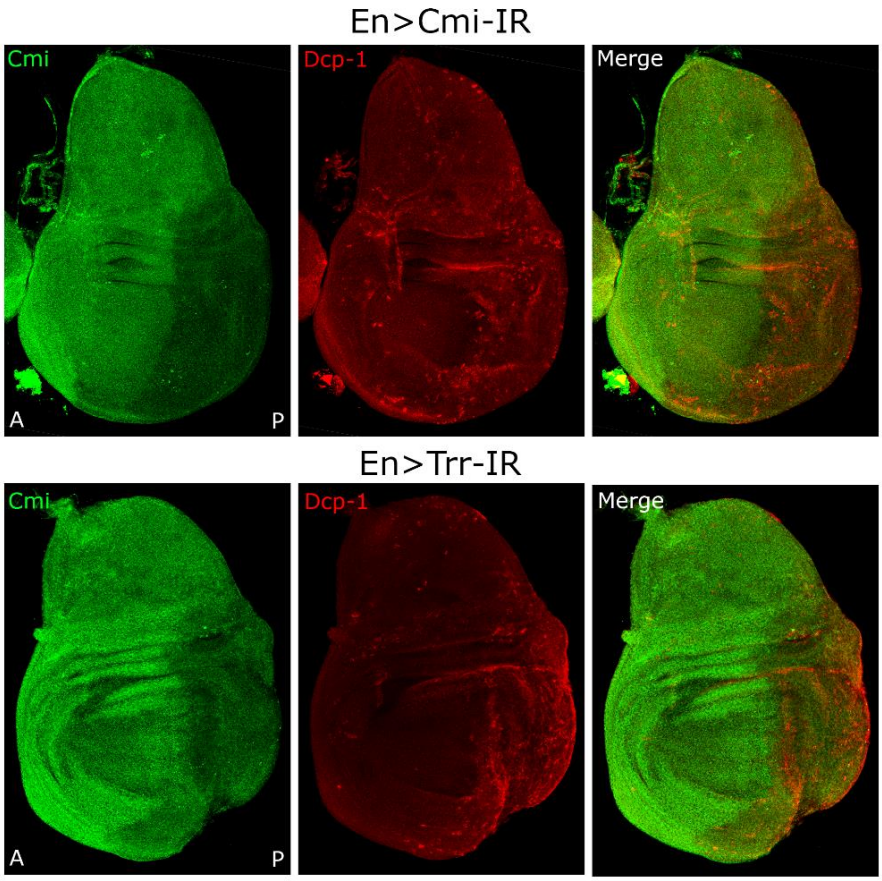

Figure 6. Knockdown of *Cmi* or *trr* in the Wing Disc Causes Increased Apoptosis. Wing discs from W3L knocking down (KD) Cmi or Trr in the posterior margin via En-Gal4 were stained for Dcp-1 (green) to marked activated effector caspase and Cmi (red) to distinguish cells experiencing KD. KD cells demonstrated higher rates of caspase activation that neighboring control tissue.

The MLR Complex is Enriched at and Regulates the Activity of Tissue-Specific bantam Enhancers

A potential regulatory target of the MLR complex is the miRNA *bantam*, the best-characterized function of which is to promote cell survival in developing tissues through translation inhibition of pro-apoptotic protein Hid [112]. *bantam* is a transcriptional target as well as feedback regulator of the Hippo, Dpp(TGF-β), and Notch pathways in Drosophila [120,122,125–127]. MLR complexes have been demonstrated to interact with and be necessary for the proper expression of the targets of these same developmental signaling pathways [48–50,56,57], suggesting that regulation of *bantam* expression by these pathways will require MLR activity. ChIP-seq data from our lab has determined that during imaginal disc development Cmi is enriched throughout the *bantam* regulatory locus, including peaks at two previously identified tissue-specific *bantam* enhancer regions. These regions are verified distal regulatory elements whose activity replicates expression patterns of *bantam* in the wing disc and in the undifferentiated eye disc, respectively [111]. Localization of MLR at these enhancer elements may imply regulatory activity, but to verify this, reporter constructs (bwe-LacZ and bee-Lacz) were used to investigate the effects of *Cmi* or *Trr* knockdown on the activity of these enhancers. Upon depletion of Cmi/Trr, *bantam* wing enhancer activity increased without alteration of its expression pattern, suggesting that the MLR complex has a role in suppressing its activity (**Fig. 7A**). The expression pattern of the *bantam* eye enhancer is dysregulated upon *Cmi*/*Trr* knockdown, demonstrating sporadic loss of activity at the anterior margin of the disc as well as ectopic more posterior activity (**Fig 7B**). This suggests that the MLR complex is necessary for correct spatiotemporal activation of this enhancer region. Altogether, these data demonstrate that the MLR complex is

recruited to tissue specific *bantam* enhancers, where it is necessary for correctly regulating the activity of those enhancers during organ development.

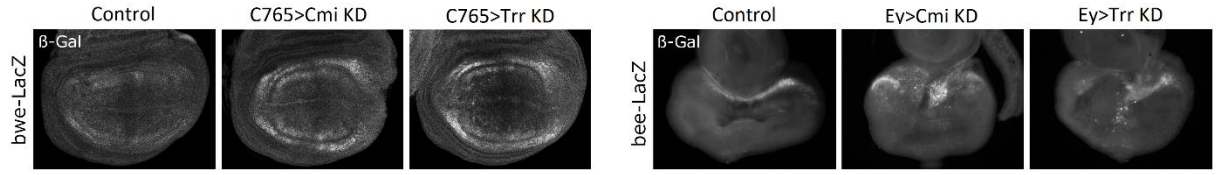

Figure 7. Tissue-specific *bantam* Enhancers are Sensitive to Levels of Cmi or Trr. A. C765-Gal4 was used to knockdown either *Cmi* or *trr* in the entire wing disc. Activity of the *bantam* wing enhancer was assayed using the bwe-LacZ reporter construct, with β-Gal expression as readout. Cmi or Trr KD increase activity of the wing enhancer. *bwe-LacZ/C765-Gal4* or *bee-LacZ/Ey-Gal4* genotypes used as controls. **B.** Ey-Gal4 was used to knockdown either *Cmi* or *trr* in the entire eye pouch. Activity of the *bantam* eye enhancer was assayed using the bee-LacZ reporter construct, with β-Gal expression as readout. Cmi or Trr KD dysregulate activity of the eye enhancer.

The MLR Complex Functions to Suppress bantam Expression During Wing Development

Suppression of the *bantam* wing enhancer by the MLR complex suggests that a role of the complex in the wing disc is to reduce expression of the *bantam* miRNA. To test this, a *bantam* sensor GFP (*ban*sens-GFP) inverse reporter construct was used. In cells containing the sensor, GFP acts as an inverse readout of *bantam* expression/activity; the higher the expression of GFP, the lower the levels of *bantam* [112]. Using the En-Gal4 driver, *Cmi* or *Trr* was knocked down only within the posterior margin of the wing disc, leaving the anterior as an internal control expressing wild-type *Cmi*/*Trr* levels. The sensor demonstrates that *bantam* levels are significantly higher (as evidenced by lower GFP) in the knockdown tissue as compared to the control, verifying the MLR complex's role is suppressing *bantam* transcription (**Fig. 8**).

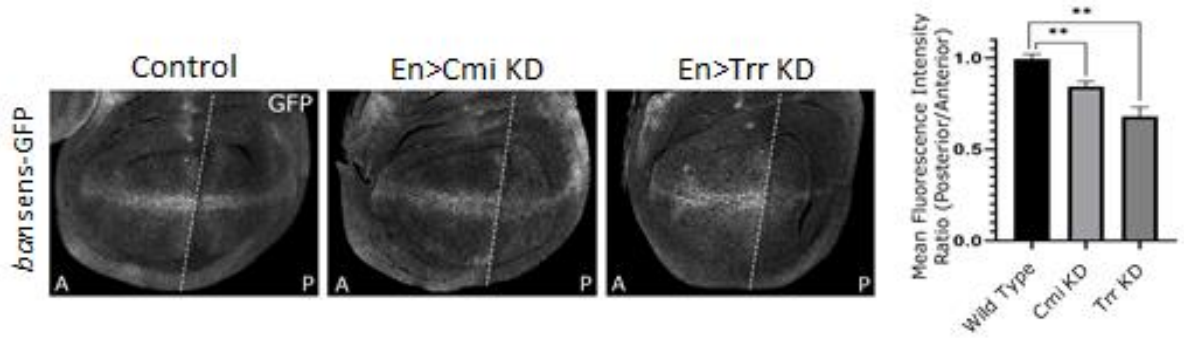

Figure 8. Knockdown of Cmi or Trr Increases *bantam* Expression in the Wing Disc. En-Gal4 was used to knockdown either Cmi or Trr in the posterior margin of the wing disc. *ban*sensGFP was used an inverse readout of *bantam* activity. *bansens-GFP*/*En-Gal4* genotype used as control. (Left) Wing tissue knocking down either Cmi or Trr displayed significantly lower GFP expression (higher *bantam*) than neighboring control tissue. (Right) Quantification of ratio of GFP expression between knockdown and control tissue; cohorts of six wing disc per genotype. Statistical significance measured by Student's T Test.

We have previously demonstrated that modulating levels of Cmi in the wing disc causes vein formation defects in adult organs [11]; Cmi KD leads to distal vein retraction (**Fig. 9B**) while *Cmi* overexpression (Cmi OE) causes shrunken wings displaying vein end splitting and ectopic vein formation (**Fig. 9C**). We subsequently determined that the MLR complex is required to positively regulate Dpp(TGF-β) signaling activity in the wing disc and that these phenotypes are caused by dysregulated Dpp signaling [52]. As *bantam* acts as a negative feedback regulator of Dpp signaling via inhibition of Mad translation [125], it is likely that proper regulation of *bantam* expression by the MLR complex may also be necessary for proper wing vein formation. To investigate this, I performed genetic interaction experiments between Cmi and *bantam*. In short, if a phenotype caused by altering one factor is sensitive to alterations of a second factor, the two are deemed to likely interact mechanistically. This synergy or dysergy demonstrates that the factors have a functional relationship in the context of the analyzed phenotype. To perform this, I modulated *bantam* levels in the wing via Gal4-driven expression of a *bantam*-

specific miRNA sponge (*ban*sponge) or a *bantam* overexpressing construct (*bantam* OE). When *bantam* levels are reduced in the background of *Cmi* knockdown, the vein retraction phenotype is completely suppressed (**Fig. 9E, Table 3**). *bantam* reduction in the background of Cmi OE instead enhances the ectopic vein phenotype (**Fig. 9F, Table 3**). If *bantam* is overexpressed alongside Cmi KD, vein retraction increases (**Fig 9H, Table 3**). Combined *bantam* OE and Cmi OE, while not clearly suppressing ectopic vein formation, does rescue wild type wing size (**Fig. 9I, Table 3**). These data demonstrate an inverse functional relationship between Cmi and *bantam* levels in the wing, verifying the expression data and demonstrating that not only does the MLR complex function by repressing *bantam* expression in the wing, but that this suppression has important developmental consequences.

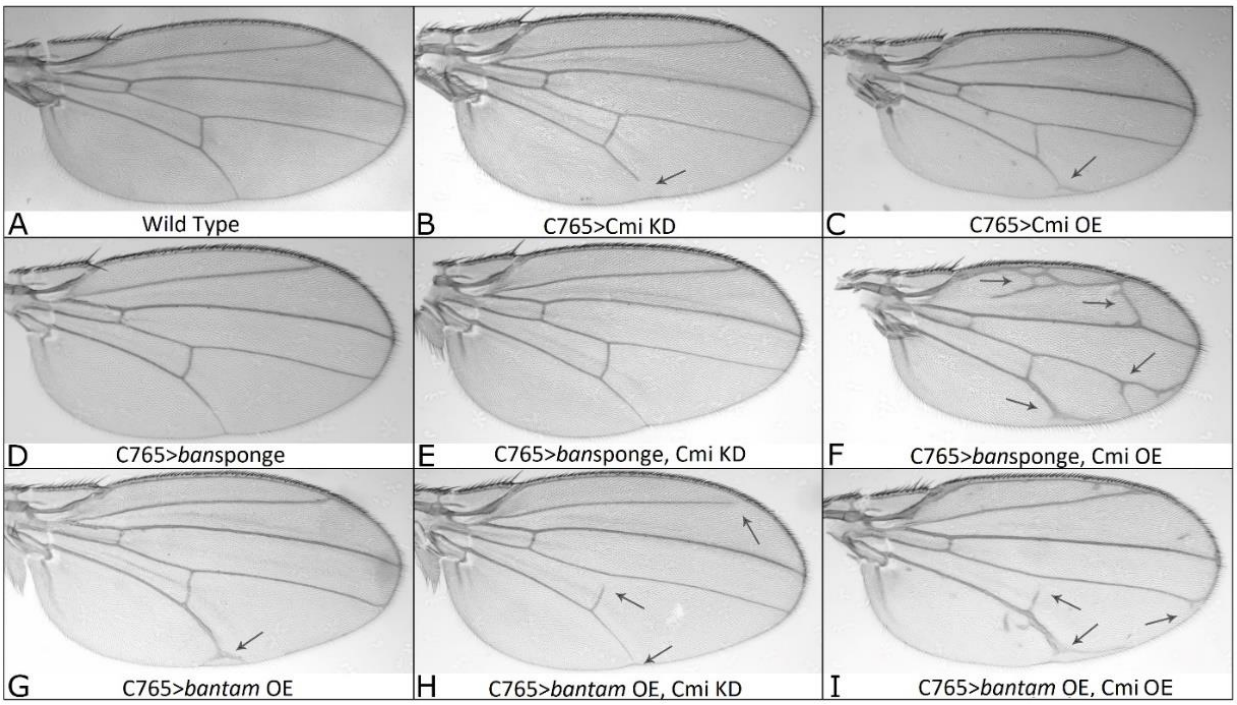

Figure 9. The MLR Complex and *bantam* Phenotypically Interact in the Wing. All constructs driven in the entire wing using C765-Gal4. **A.** Wild type control wing (OregonR strain). **B.** Cmi knockdown (KD) causes slight vein retraction. **C.** Cmi overexpression (OE) causes slight vein end splitting and shrunken wings. **D.** Reduction of *bantam* activity via the *ban*-sponge alone has no phenotypic effect. **E.** *bantam* reduction suppresses the Cmi KD phenotype. **F.** *bantam* reduction enhances the Cmi OE phenotype **G.** *bantam* OE alone causes slight vein end splitting. **H.** *bantam* OE enhances the Cmi KD phenotype. **I.** *bantam* OE rescues wing size in Cmi OE wings but vein defects remain. (*N > 50 for each genotype*)

Table 3. Wing Phenotype Scoring. All constructs driven in the entire wing using C765-Gal4. Adult wings of each genotype were scored according to wing vein patterning. The N value refers to number of individual wings scored and the value in each table represents a percentage of the population displaying a phenotype.

	Wild Type	C765>Cmi KD	C765>Cmi KD,*bantam* OE	C765>Cmi KD,*ban* sponge	C765>Cmi OE	C765>Cmi OE,*bantam* OE	C765>Cmi OE,*ban* sponge	C765>*bantam* OE	C765>*ban* sponge
	N = 121	N = 51	N = 284	N = 133	N = 128	N = 132	N = 502	N = 80	N = 601
Wild Type	100	90	86	100			6		72
L2 Retraction			5						
L5 Retraction		4	6						
PCV Retraction		10	12						
L4 Branching					9	100	54		
L5 Branching					91	100	94	100	
Ectopic PCV					5				
PCV Branching							4		28
PCV Loss						49			

The Effects of Cmi or Trr Knockdown in the Developing Eye are Sensitive to bantam Levels

To begin investigating if the MLR complex plays a similar repressive role on *bantam* expression in the eye as in the wing, I performed genetic interaction experiments modulating *bantam* levels in the eye in the background of Cmi or Trr KD (**Fig. 10A**). Overexpression of *bantam* significantly enhances the rough and shrunken eye phenotype caused by Cmi or Trr KD, while *bantam* reduction suppresses the phenotype. This inverse relationship is demonstrated not only by comparing the phenotypes of entire genetic populations, but also by comparing individual compound eyes at the ommatidial level (**Fig. 10B**). This inverse functional relationship between Cmi/Trr and *bantam* suggests that the MLR complex is necessary for negatively regulating *bantam* expression in the eye, just as in the wing. Importantly, overexpression of *bantam* alone phenocopies the rough and shrunken eyes seen in MLR-depleted eyes (**Fig. 10A-B**), suggesting that altered *bantam* levels may be causal to the phenotype.

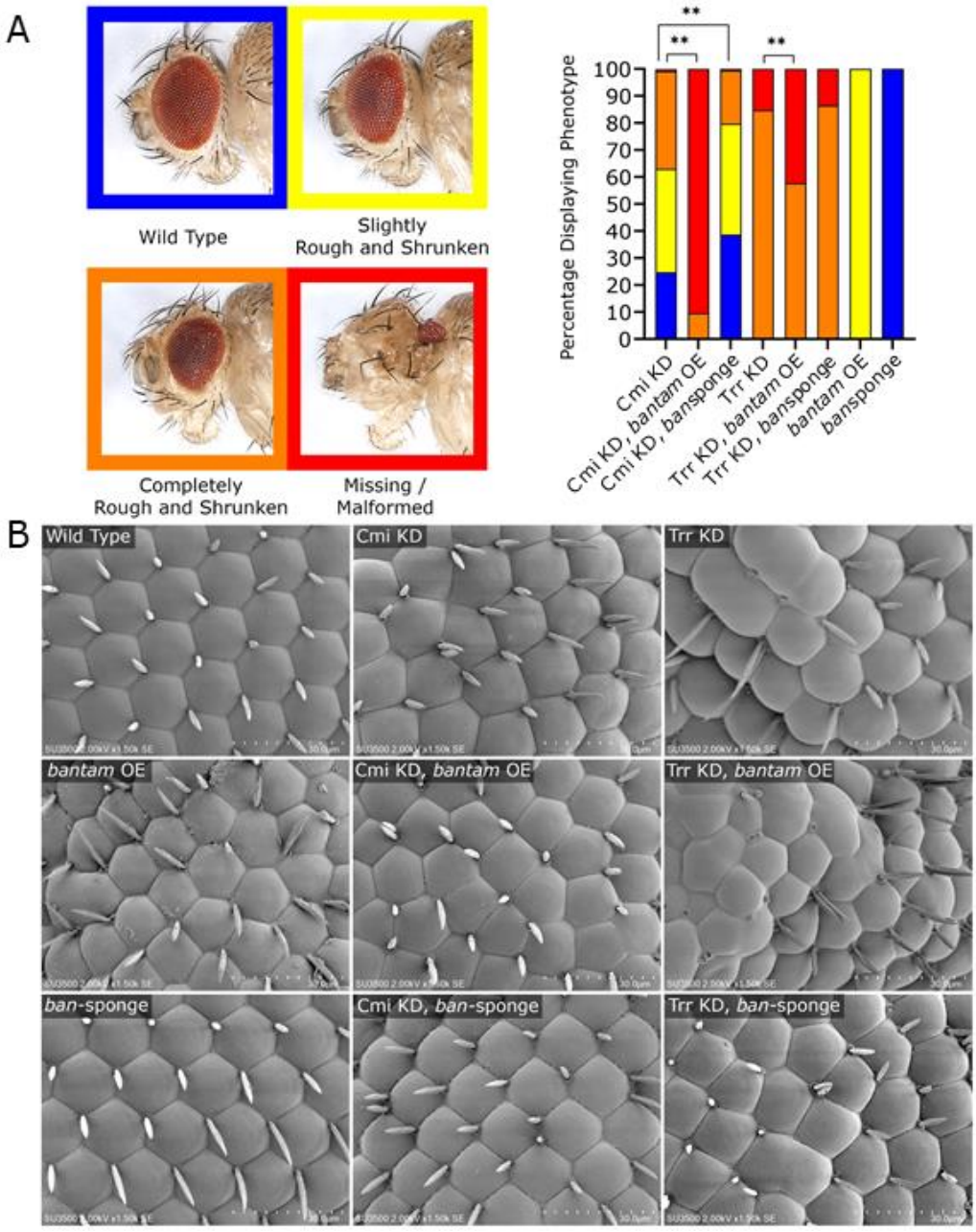

Figure 10. The MLR Complex and *bantam* Phenotypically Interact in the Eye. All constructs driven in the entire eye pouch using Ey-Gal4. **A.** (Right) All genotypes scored for penetrance and expressivity according to (Left) phenotype severity. (*N > 100 for each genotype; statistical significance measured by Chi Square Test for Population Variance; ** = p<0.01.*) **B.** All genotypes were examined for severity of ommatidial patterning defects via SEM. OregonR strain used as wild type control. **A-B.** *bantam* overexpression (OE) alone causes a rough and shrunken phenotype similar to Cmi or Trr knockdown (KD). *bantam* OE enhances the Cmi/Trr KD phenotype. Reduction of *bantam* activity via the *ban*-sponge suppresses the Cmi/Trr KD phenotype.

The MLR Complex is Required in Undifferentiated Eye Tissue for Regulation of *bantam* Expression upon Differentiation

To investigate if *bantam* levels are sensitive to MLR subunit knockdown in the eye disc as they are in the wing disc, I assayed ban*sens* expression upon Cmi or Trr KD. The eye disc is a more heterogenous tissue than the wing disc, not only containing cells destined for different lineages (eye, antennal, head capsule, etc.) but also cells at different stages of differentiation and development [101,103]. The morphogenetic furrow, which bisects the eye pouch, marks the boundary between undifferentiated cells to the anterior and differentiating cells to the posterior [175]. To take advantage of this unique developmental system and explore whether the MLR complex has similar effects on *bantam* expression in eye cells at different stages of differentiation, multiple Gal4-drivers were used to drive either Cmi KD or Trr KD in specific cell populations (**Fig. 11A**). Ey-Gal4 was used to knockdown within the eye pouch but not the antennal section; GawB69B-Gal4 was used to knockdown ubiquitously throughout the entire disc; DE-Gal4 was used to knockdown within the dorsal half of the eye pouch, leaving the ventral as an internal wild type control; GMR-Gal4 was used to knockdown only in differentiating tissue posterior to the morphogenetic furrow. In wild type eye discs, *bantam* levels remain relatively high in undifferentiated tissue; once differentiation commences, *bantam* is downregulated in proneuronal cells at the center of each developing ommatidia and upregulated in the interommatidial cells bordering the compound eye units (**Fig. 11B**) [176]. Knockdown by any Gal4 driver of either *Cmi* or *Trr* in the undifferentiated cells anterior to the morphogenetic furrow does not visibly alter bantam expression in those cells (**Fig. 11C-F**). However, in Ey-Gal4, GawB69B-Gal4, and the dorsal half of DE-Gal4 discs, depletion of Cmi or

Trr appears to cause downregulation of *bantam* in differentiating cells, as evidenced by higher GFP sensor signal posterior to the furrow (**Fig. 11C-E**). These results suggest that the MLR complex in dispensable for regulating *bantam* expression in undifferentiated eye tissue, yet is required for proper *bantam* expression upon differentiation. Given this, it would be assumed that knockdown of Cmi or Trr within the differentiating cells alone via GMR-Gal4 would produce a similar effect. Surprisingly, this instead had no effect, matching wild type expression of *bantam* (**Fig. 11F**). This indicates that the MLR complex has regulatory function in undifferentiated eye cells necessary for proper bantam expression upon differentiation, yet is unnecessary for regulating bantam levels once differentiation has commenced.

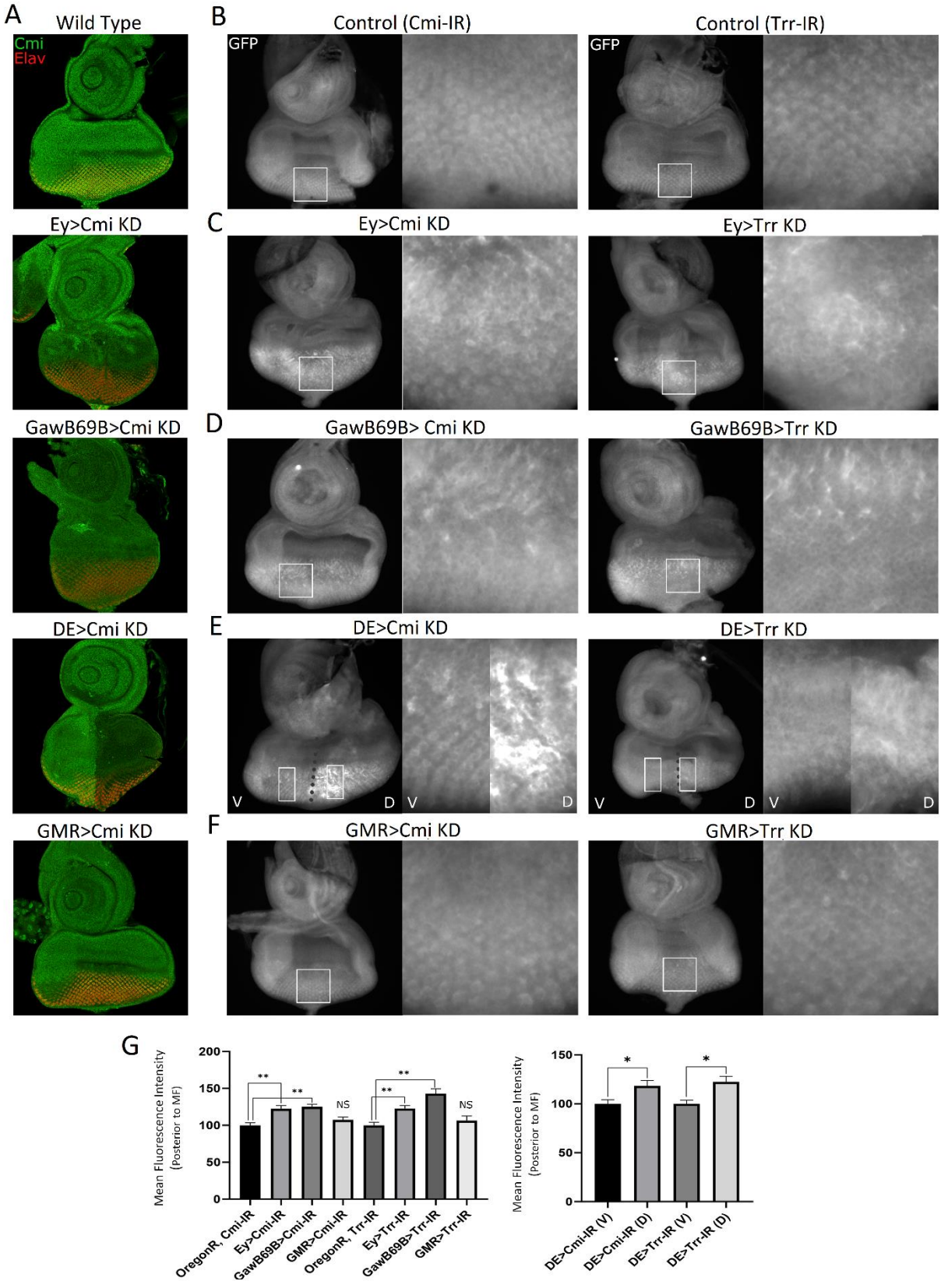
A
Wild Type
Cmi
Elav
B
Control (Cmi-IR)
GFP
Control (Trr-IR)
GFP
Ey>Cmi KD
C
Ey>Cmi KD
Ey>Trr KD
GawB69B>Cmi KD
D
GawB69B> Cmi KD
GawB69B>Trr KD
DE>Cmi KD
E
DE>Cmi KD
DE>Trr KD
V
D
V
D
V
D
V
D
GMR>Cmi KD
F
GMR>Cmi KD
GMR>Trr KD
G
Mean Fluorescence Intensity (Posterior to MF)
200
150
100
50
0
**
**
NS
**
**
NS
OregonR, Cmi-IR
Ey>Cmi-IR
GawB69B>Cmi-IR
GMR>Cmi-IR
OregonR, Trr-IR
Ey>Trr-IR
GawB69B>Trr-IR
GMR>Trr-IR
Mean Fluorescence Intensity (Posterior to MF)
150
100
50
0
*
*
DE>Cmi-IR (V)
DE>Cmi-IR (D)
DE>Trr-IR (V)
DE>Trr-IR (D)

Figure 11. The MLR Complex Regulates *bantam* Expression in the Differentiating Eye. A. Cmi was knocked down using various Gal4 drivers to visualize driver expression pattern. Expression pattern of these drivers is visualized by staining for Cmi (green) and Elav (red), which labels proneuronal cells posterior to the morphogenetic furrow. **B-F.** *ban*sensGFP was used an inverse readout of *bantam* activity in W3L eye discs. Magnified views of developing ommatidia posterior to the furrow are displayed to the right of each example disc. bantam activity in the undifferentiated anterior tissue anterior to the furrow remain unchanged in all genotypes. **B.** In control discs, bantam activity is relatively high anterior to the furrow (low GFP) and lower posterior (high GFP). **C.** Ey-Gal4 was used to drive Cmi or Trr KD in the entire eye pouch. GFP expression increases in differentiating tissue, demonstrating decrease in *bantam* level. **D.** GawB69B was used to drive KD ubiquitously. Again, GFP expression increases in differentiating tissue. **E.** DE-Gal4 was used to drive KD only within the dorsal half of the eye pouch. Within dorsal differentiating cells, GFP expression increases. **F.** GMR-Gal4 was used to drive KD only within the differentiating cells posterior to the furrow. *ban*sens-GFP matched wild type. **G.** Mean fluorescence intensity anterior to the furrow was quantified from cohorts of each genotype (*N ≥ 10 for all genotypes; statistical significance measured by Student's T Test; * = p≤0.05, ** = p≤0.01, NS = not significant.*)

The MLR Complex is Required to Regulate *bantam* Differentially Depending on the Context of Cell Fate

The apparent downregulation of bantam expression observed in differentiating eye cells does not appear to be uniform, but rather manifested as sporadic increases in ban*sens*-GFP expression in cells posterior to the morphogenetic furrow (**Fig 11C-E**). This suggests that the effects of MLR depletion on *bantam* expression varies by cell type. I therefore used confocal microscopy focusing on the developing ommatidia to determine in a cell-specific manner how *bantam* levels are affected by loss of MLR complex activity. I drove knockdown of either *Cmi* or *Trr* throughout the eye pouch using Ey-Gal4 and stained for proneuronal protein Elav to distinguish cell fate among the differentiating tissue. As a reminder, during early ommatidial differentiation cells of the proneuronal cell fate (developing into photoreceptors) are Elav-positive and cells of the interommatidial cell fate (developing into structural pigment cells) are

Elav-negative. In wild type tissue, *bantam* levels are relatively low in proneuronal cells and relatively high in neighboring interommatidial cells (**Fig. 12**). Upon Ey-Gal4-driven depletion of Cmi or Trr, *bantam* is simultaneously upregulated in proneuronal ommatidial cells and downregulated in interommatidial cells. These results reveal that the MLR complex is utilized to regulate the expression of a single transcriptional target in opposite direction depending on the context of cell fate. The sensitivity of the rough and shrunken eye phenotype to *bantam* levels suggests that this regulatory activity is also critical for proper organ development.

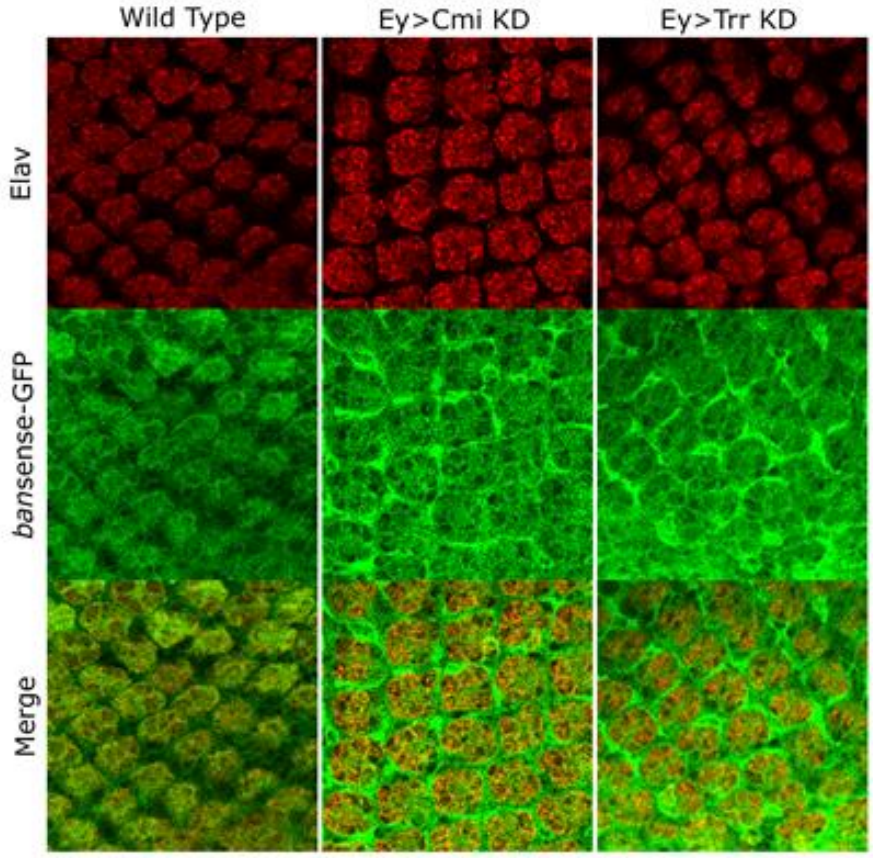

Figure 12. The MLR Complex is Required to Regulate *bantam* in Differentiating Ommatidia According to Cell Fate. Developing ommatidia in eye discs were stained for pro-neuronal marker Elav (red) and *ban*sensGFP (green). Control organs (genotype *OregonR/Ey-Gal4*) demonstrate colocalization of GFP and Elav. Upon Ey-Gal4-driven Cmi or Trr KD, changes in bantam expression vary by cell fate. In proneuronal cells *bantam* activity is increased (lower GFP), and in interommatidial cells *bantam* activity is decreased (higher GFP).

Caspase Activation in MLR-Depleted Eye Discs is Not Causal to Rough and Shrunken Eyes

I originally investigated *bantam* as a candidate regulatory target of the MLR complex whose dysregulation may lead to the observed apoptotic phenotype. However, my results demonstrate that this cannot be the case: apoptosis is induced in undifferentiated eye cells upon Ey-Gal4-driven knockdown of *Cmi* or *Trr* (**Fig. 6**), yet this reduction of MLR activity has no effect on *bantam* expression in those cells (**Fig. 11**). Despite this conclusion, to better characterize the nature of this apoptotic effect I sought to determine if the caspase activation caused by Cmi or Trr KD is sensitive to *bantam* levels, just as the adult rough and shrunken eye phenotype is (**Fig. 10**). This was again accomplished via genetic interaction tests between Cmi/Trr and *bantam*. Just as in the adult eye, the Cmi/Trr KD phenotype is enhanced by overexpression of *bantam* and suppressed by *bantam* decrease via the *ban*sponge (**Fig. 13A-C**). These results further support the assumed causal link between aberrant cell death during development and the shrunken and mispatterned adult organs, as both effects are similarly sensitive to *bantam* levels. In order to verify this, apoptosis was directly suppressed via expression of p35, a baculovirus substrate inhibitor of caspases including Dcp-1 [177,178]. If the apoptosis resulting from MLR subunit depletion is causal the rough and shrunken eyes, then caspase inhibition by p35 would suppress the adult phenotype. While 35 expression successfully suppresses caspase cascade in a Cmi/Trr KD background (**Fig. 13D**), it enhances the rough and shrunken phenotype (**Fig. 13E**). These data demonstrate that the apoptotic phenotype and the malformed eye phenotype are two mechanistically separate results of loss of MLR complex activity.

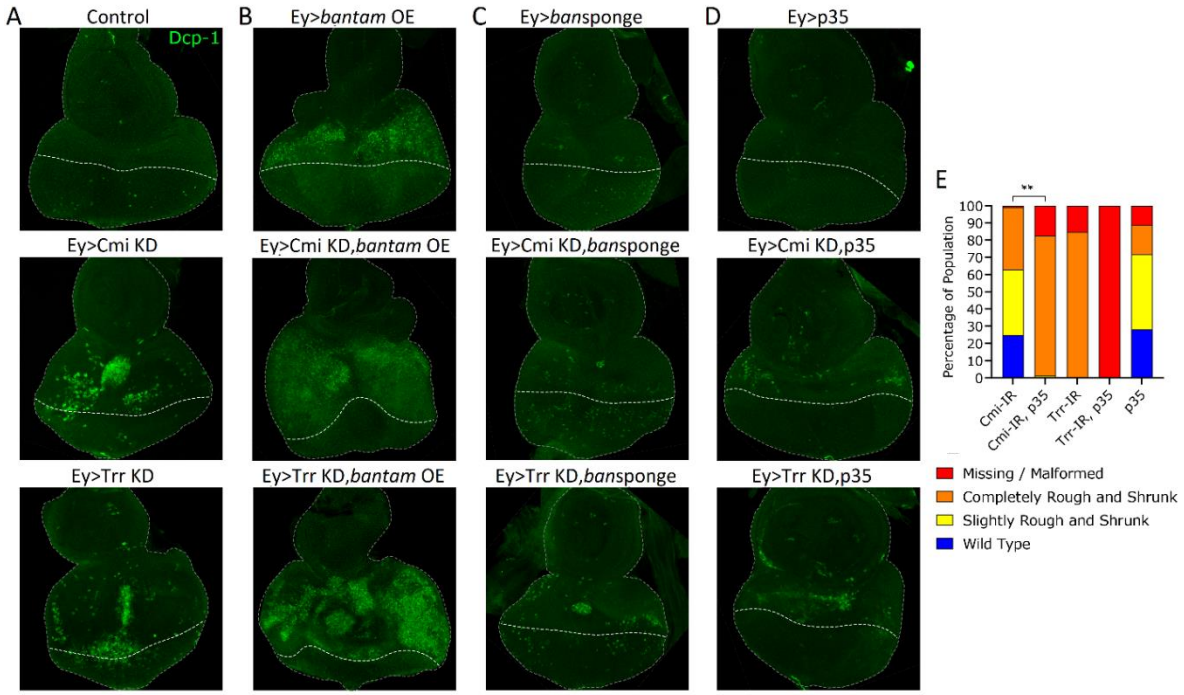

Figure 13. MLR Complex Depletion-associated Caspase Activation is Sensitive to *bantam* Levels, Mechanistically Separate from Rough and Shrunken Phenotype. All constructs driven in the entire eye pouch using Ey-Gal4. *OregonR-Ey-Gal4* genotype used as control. **A-D.** W3L eye disc were stained for activated caspase Dcp-1. The morphogenetic furrow is marked by the white dotted line; each disc is outlined in a thinner gray dotted line. **A.** As shown previously, Cmi or Trr KD causes increased caspase activity in undifferentiated eye cells. **B.** *bantam* OE alone causes generalized increase in caspase activity in undifferentiated eye cells and enhances the effects of Cmi or Trr KD. **C.** *bantam* reduction via *ban*-sponge alone has no effect on caspase activation, yet suppresses the effects of Cmi or Trr KD. **D.** Expression of pan-caspase inhibitor p35 suppresses the caspase activation phenotype of Cmi or Trr KD. **E.** Expression of pan-caspase inhibitor p35 enhances the rough and shrunken eye phenotype of Cmi or Trr KD. (*N > 100 for each genotype except Trr-IR,p35 with N = 10; statistical significance measured by Chi Square Test for Population Variance; ** = p<0.01*)

As an additional observation, it is well documented that overexpression of *bantam* in the eye disc suppresses developmental or induced apoptosis [112]. However, my results demonstrate that Ey-Gal4-driven *bantam* OE results in a significant induction of effector caspase activity, restricted to the undifferentiated tissue anterior to the furrow (**Fig. 13B**).

Unlike caspase activation associated with Cmi/Trr KD, this effect is not concentrated but widespread, suggesting different underlying mechanisms.

The Effects of Cmi or Trr Knockdown in the Developing Eye are Sensitive to Notch Signaling Activity

The localized concentration of caspase activation in Cmi/Trr-knockdown eye discs (**Fig. 6**) suggests that the effect is not due to dysregulation of general survival machinery, but rather the dysregulation of factors specific to that location, such as developmental signaling pathways. The fact that the phenotype is sensitive to *bantam* levels (**Fig. 13**) suggests that the effect involves a target of the miRNA, such as the Notch inhibitor Numb [126]. It has been demonstrated both in Drosophila imaginal discs and in human cell lines that MLR complexes are recruited to Notch targets genes and are necessary for proper regulation of those targets [55–57]. During eye development, Notch signaling is activated on the dorsal-ventral midline and functions to promote survival of the undifferentiated cells through expression of its target *Eyg* [105]. Therefore, it is likely that the MLR complex is necessary for regulating Notch's pro-survival activity in the undifferentiated eye, and that apoptosis occurs when and where this function is lost. Beyond this, Notch signaling has further function during differentiation and compound eye development, including photoreceptor patterning, cell fate adoption, and regulation of the final round of mitosis [179–181]. Therefore, dysregulation of Notch signaling may also underlie the patterning and size defects in the adult eye caused by depletion of MLR complex activity. To begin investigating this, I performed genetic interaction experiments between MLR subunits and multiple components of Notch signaling in the developing eye. These included reducing Notch signaling via either hypomorphic Notch mutant ($N^{spl\text{-}1}$) or overexpression of inhibitor

Numb, as well enhancing Notch signaling via either knockdown of Numb expression of overexpression of Notch target Eyg. In the background of Cmi or Trr KD in the eye, both the N^{spl-1} mutant and Numb overexpression significantly suppressed the rough and shrunken phenotype, while both Numb knockdown and Eyg overexpression enhanced the phenotype (**Fig. 14**). Notably, either Numb knockdown or Eyg overexpression alone phenocopied the rough and shrunken phenotype found in MLR-depleted animals. These results strongly suggest not only that Notch signaling and MLR activity have an inverse relationship during compound eye development, but also that overactivation of Notch signaling may be the mechanism underlying the rough and shrunken adult eye phenotype.

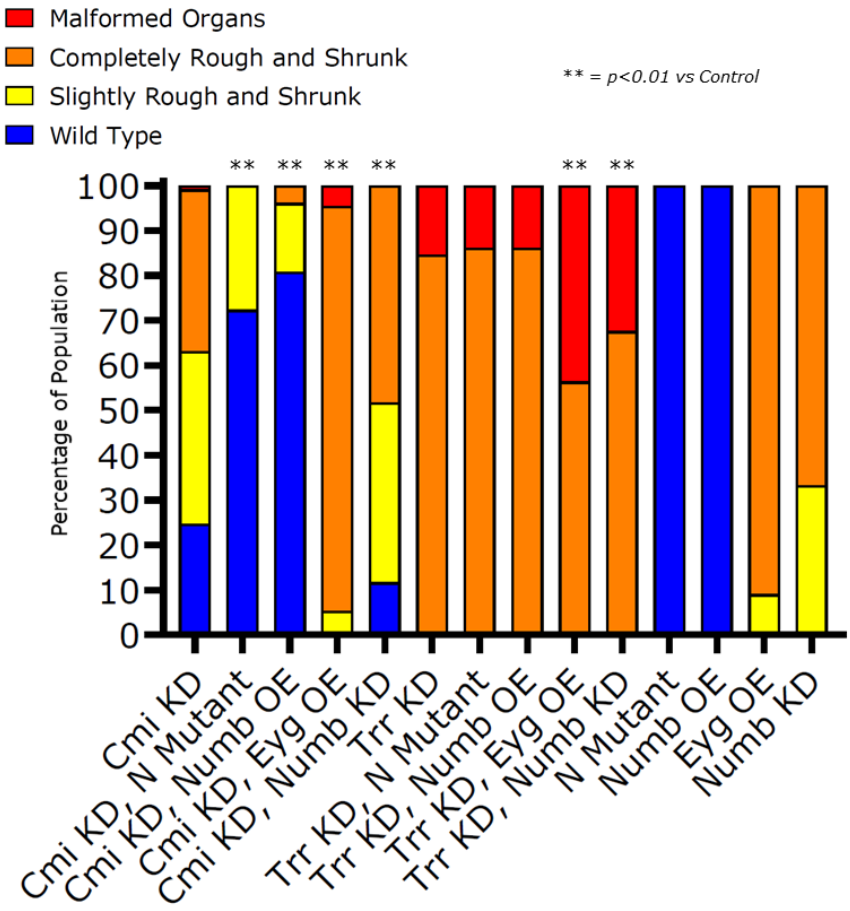

Figure 14. The MLR Complex and Notch Phenotypically Interact in the Eye. All constructs driven in the entire eye pouch using Ey-Gal4. Reduction of Notch signaling activity via hypomorphic Notch mutant or OE of Notch inhibitor Numb suppress the rough and shrunken phenotype of Cmi or Trr KD. Increase of Notch signaling activity via OE of Notch eye-specific target Eyg or KD of Notch inhibitor Numb enhances the rough and shrunken phenotype of Cmi or Trr KD as well as phenocopies the effects alone. (*N > 100 for each genotype; statistical significance measured by Chi Square Test for Population Variance, ** = p<0.01.*)

Knockdown of Cmi or Trr Reduces Notch Response Element Activation in Eye Disc

The previous interaction experiments between the MLR complex and Notch signaling suggest that the complex may function by negatively regulating Notch signaling activity during eye development. This is supported by previous reports which found that MLR complexes suppress Notch signaling through downregulation of its machinery, including Notch, RBPJ/Su(h),

Hes1/4, and Jag1/2 [55,56,58,182]. To determine if depletion of Cmi or Trr leads to increased Notch signaling activity, I utilized a Notch Response Element GFP recombinant reporter construct (NRE-GFP) created by fusing the regulatory region of E(spl)mβ (a classic Notch target) to a GFP coding region. Expression of NRE-GFP in the eye disc demonstrated a solid pattern of activation on the dorsal-ventral midline of the eye pouch, as has been previously demonstrated [183,184] (**Fig. 15**). Unexpectedly, knockdown of either Cmi or Trr resulted in reduction of NRE-GFP activity, particularly in undifferentiated cells, suggesting that the MLR complex may be used to positively regulate Notch targets in this tissue at this developmental stage. While counter to the evidence from genetic interaction in the adult eye, reduction of Notch activity upon Cmi/Trr depletion does support the hypothesis that suppressed Notch signaling underlies the apoptotic phenotype in the undifferentiated eye.

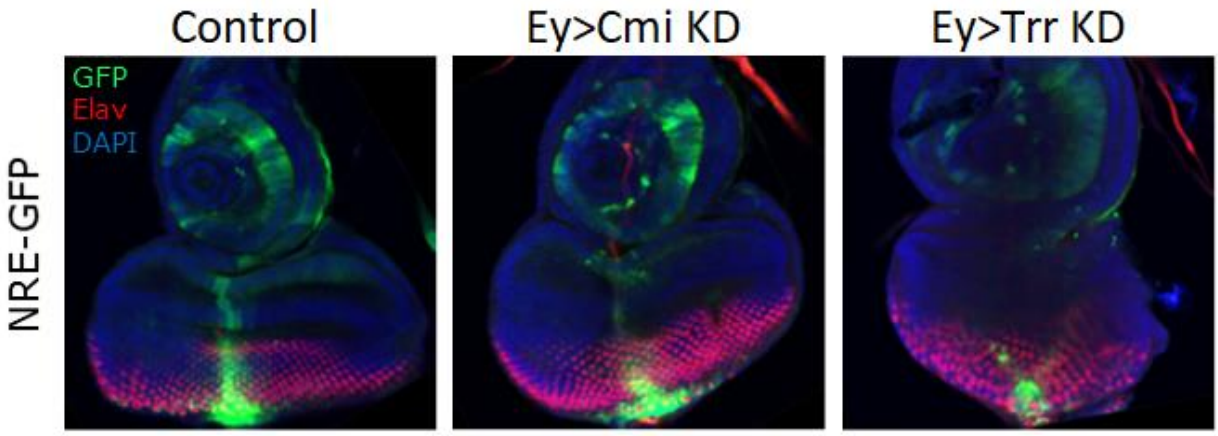

Figure 15. The MLR Complex is Required for Activation of Notch Targets in the Eye Disc. Activity of Notch regulatory targets was simulated using a Notch Response Element reporter construct with GFP as a readout (NRE-GFP) (green). Elav (red) distinguishes differentiating cells posterior to the morphogenetic furrow. *OregonR/NRE-GFP* genotype used as control. Upon Cmi or Trr KD, NRE-GFP signal decreases, particularly in undifferentiated eye cells.

Synthesis and Storage of Triglycerides in the Fat Body is Not Sensitive to Cmi Levels

The development of white adipose tissue in the mouse is suppressed by either the loss Kmt2c enzymatic activity or knockout of *Kmt2d* in fat precursor cells [29,30]. Murine brown pre-adipocytes deficient in both *Kmt2c* and *Kmt2d*, when induced to differentiate, demonstrate severely reduced adipogenic potential and inability to induce adipose-specific genes; Kmt2d interacts with PPARγ and is necessary for properly priming critical differentiation-associated enhancer regions [29]. These investigations conclude that MLR complexes are necessary for fat tissue development through regulating the activation of lineage-specific reprogramming during differentiation. While Drosophila does not contain a clear ortholog to PPARγ in its genome, I sought to determine if the MLR complex's role in regulating fat tissue development is conserved in the fruit fly.

In order to investigate this, I modulated MLR activity in the fat tissue through increase or decrease of Cmi protein levels by either overexpressing Cmi (Cmi OE) via expression of a full-length Cmi cDNA or the Cmi-specific RNAi hairpin (Cmi KD), respectively. The expression of either construct was driven by Lsp2-Gal4, which is expressed strongly and exclusively within the fat body tissue of the larva, pupa, and adult. Our previous studies in the wing suggest that knockdown or overexpression of Cmi is an efficient method for decreasing or increasing MLR complex activity, respectively; additionally, loss of Cmi has proven to result in generally less severe outcomes than loss of Trr, ideal for investigation of interacting factors. Therefore, no other subunit of the MLR complex, including Trr, was modulated in these studies. Neither fat body-specific Cmi OE nor Cmi KD resulted in any clear defects in fat body development or size when compared in larvae of similar developmental stages. During the larval stages of the

animal, the fat body functions to store nutrient-derived energy as TAGs to power metamorphosis. To determine if altered Cmi levels affected the fat body's ability to synthesize and store TAGs, I assayed whole-animal TAG levels at the final larval stage, the wandering third instar larva. Neither Cmi KD nor Cmi OE affected the animal's ability to store fat in its adipose tissue (**Fig. 16**).

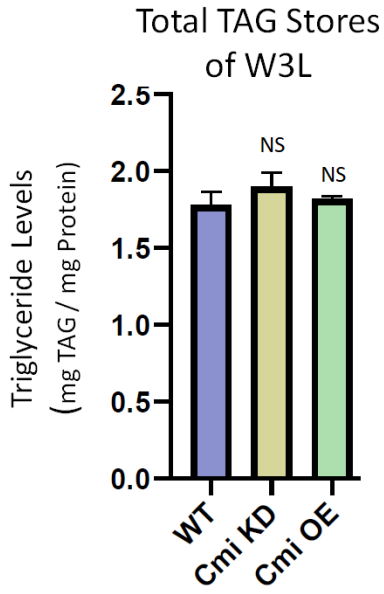

Figure 16. Cmi Knockdown or Overexpression has No Effect on Larval Triglyceride Storage. Cmi KD and Cmi OE constructs driven in the fat body using Lsp2-Gal4. *OregonR/Lsp2-Gal4* genotype used as wild type. Representative cohorts of wandering third instar larvae (W3L) were assayed for total (TAG) content. Neither Cmi KD nor Cmi OE has significantly different TAG stores than control. (*N = 4 for each genotype; statistical significance measured by Student's T Test, NS = not significant*)

Cmi Overexpression in the Fat Body Causes Metamorphosis Lethality at 29°C

While Drosophila stores TAGs during the larval periods, it uses that stored fat as its main energy source as a pupa undergoing metamorphosis, during which time it has no access to environmental nutrients [185]. In order to investigate if this process is sensitive to MLR activity, I compared Cmi KD and Cmi OE animals' survival and eclosion rates compared to control. At

25°C, Cmi level has no effect on the animal's ability to successfully complete metamorphosis as a fully-formed adult (**Table 4**). The organism's metabolic rate, and therefore the rate of depletion of stored TAGs, is temperature-sensitive: the higher the temperature, the higher the metabolic rate [185]. I took advantage of this ability to modulate metabolic rate and allowed the animals to undergo metamorphosis at the stressful temperature of 29°C; under these conditions, while control and Cmi KD pupae were able to successfully complete metamorphosis, no Cmi OE pupae survived (**Table 4**). Instead, each animal died as a fully-formed pharate inside the pupal case before beginning eclosion, what I term "pharate lethal" (**Fig. 17A**). This suggests that there may be a point during metamorphosis in Cmi OE animals that is sensitive to 29°C and causes eventual developmental arrest and/or lethality. To investigate this, I exposed pupae to two different conditions: undergoing early metamorphosis at 25°C before being transferred to 29°C for late metamorphosis, or vice versa. Interestingly, neither group of Cmi OE animals demonstrated greater lethality. Instead, while all animals of both groups failed to survive metamorphosis, approximately 25% of each group were able to begin the process of eclosion but died before completion, what I term "eclosion lethal" (**Fig. 17B**). Three Cmi OE animals under these conditions were observed during the process of eclosion and manually removed from the pupal case, fully intact and alive. These animals all died within hours after removal, demonstrating that lethality is not inherent to physical inability to complete eclosion. These results suggest that the susceptibility to pupal death at 29°C in Cmi OE animals is not due to metamorphic defect at a particular developmental point, but rather a general sensitivity during the process of metamorphosis.

Table 4. Cmi overexpression in the Fat Body Causes Metamorphic Lethality at 29°C. Cmi KD and Cmi OE constructs driven in the fat body using Lsp2-Gal4. Pupae underwent metamorphosis at 25°, 29°, or switched between the two temperatures halfway through. Control and Cmi KD pupae successfully completed metamorphosis and eclosed as healthy adults at all temperatures. At 29°, all Cmi OE pupae were scored as Pharate Lethal (PL). At either temperature switch condition, approximately 75% of Cmi OE pupae were scored as LPL, while the remaining 25% were scored as Eclosion Lethal (EcL).

Survival Through Metamorphosis

Metamophosis Temp. (°C)		% Survival		
Early	Late	Control	Cmi KD	Cmi OE
25	25	100	100	100
29	29	100	100	0 (PL)
25	29	100	100	0 (75% PL, 25% EcL)
29	25	100	100	0 (75% PL, 25% EcL)

PL: Pharate Lethal EcL: Eclosion Lethal

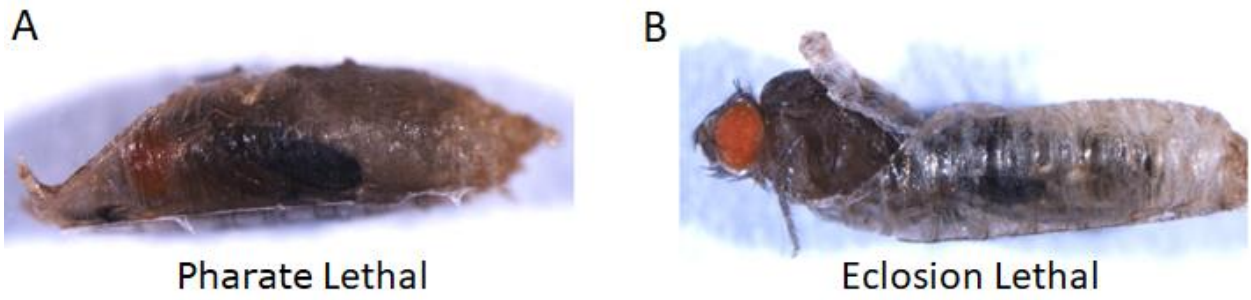

Figure 17. Examples of Lethality in Cmi OE Pupae. A. An example of the LPL phenotype, consisting of a fully-formed adult that does not begin the eclosion process. **B.** An example of the EcL phenotype, consisting of a fully-formed adult dying before completion of the eclosion process.

Cmi Overexpression in the Fat Body Increases Triglyceride Depletion During Metamorphosis

After the initial stages of pupariation, eclosion is the most energetically taxing process of metamorphosis [185]. This suggests that Cmi OE pupae do not have the energy to complete eclosion at the already metabolically stressful temperate of 29°C. To investigate this, I assayed the stored TAG levels of newly-eclosed adults to compare the total TAGs used during

metamorphosis. As demonstrated above, animals enter metamorphosis with similar levels of stored TAGs (**Fig. 16**). After metamorphosis at 25°C, while control and Cmi KD animals demonstrate similar remaining TAG levels, Cmi OE animals have significantly less (**Fig. 18**), suggesting that excess levels of Cmi in the fat body increase the rate at which TAGs are depleted during metamorphosis. This concurs with the lethality of Cmi OE animals at 29°C, suggesting that pupae at this metabolically stressful temperature deplete stored TAGs to the degree that there is not enough energy to complete eclosion and/or survive thereafter.

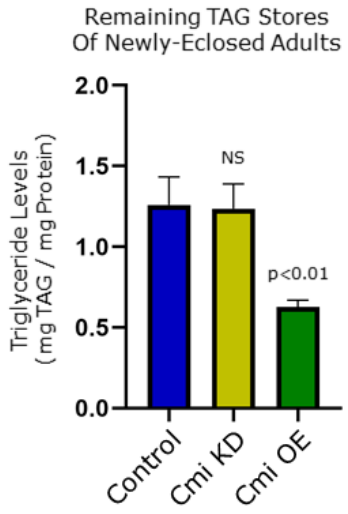

Figure 18. Cmi Overexpression in the Fat Body Increases the Rate of Triglyceride Depletion During Metamorphosis. Cmi KD and Cmi OE constructs driven in the fat body using Lsp2-Gal4. *OregonR/Lsp2-Gal4* genotype used as control. Representative cohorts of newly-eclosed adults having just completed metamorphosis at 25° were assayed for remaining triglyceride (TAG) content. While Cmi KD had no affect of TAG stores compared to control, Cmi OE animals demonstrated significantly lower levels. (*N = 4 for each genotype; statistical significance measured by Student's T Test, NS = not significant*)

Cmi Knockdown in the Fat Body Increases Starvation Resistance and Decreases Triglyceride Depletion During Starvation

Adult Drosophila also mobilize stored TAGs for energy during periods of starvation. We sought to determine if the sensitivity to Cmi level in the pupal body is present in the adult fat

body as well. To investigate this, newly-eclosed adults were placed onto starvation media, on which they have access to moisture but not to nutrients of any kind. Under these conditions, flies deplete stored TAG levels remaining after metamorphosis until death. Cmi OE adults have less stored TAGs than control and, as expected, succumb to starvation significantly earlier (**Fig. 19A**). Unexpectedly, although Cmi KD adults end metamorphosis with similar TAG levels as control, they survive starvation significantly longer. To investigate this effect, adults of each genotype were collected and assayed for TAG content during starvation. Cmi KD animals demonstrated significantly higher TAG levels than control flies at both two and three days post-starvation (**Fig. 19B**), suggesting that reduced Cmi levels in the fat body inhibit the depletion rate of TAGs during nutrient stress.

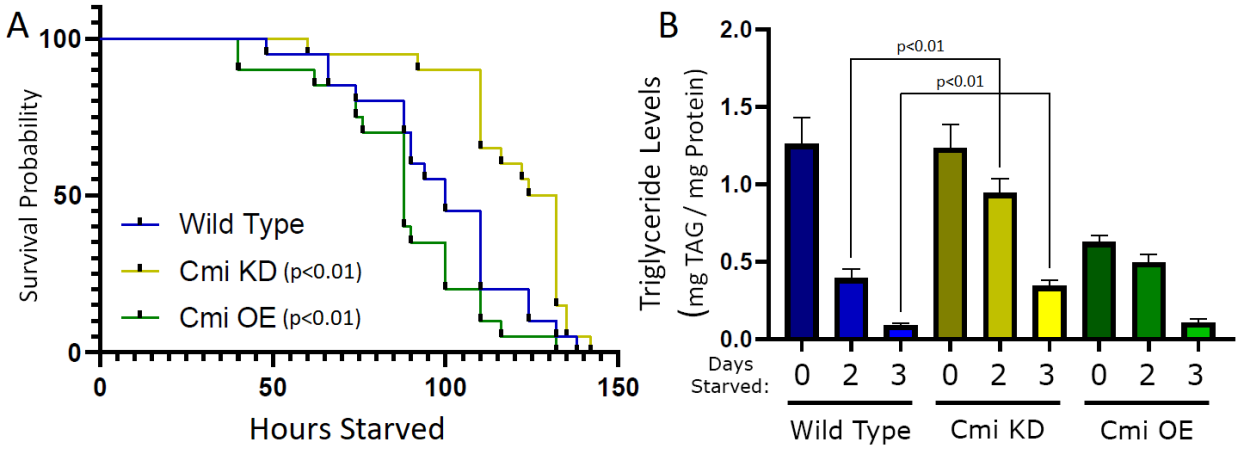

Figure 19. Cmi Knockdown in the Fat Body Decreases the Rate of Triglyceride Depletion During Starvation. Cmi KD and Cmi OE constructs driven in the fat body using Lsp2-Gal4. *OregonR/Lsp2-Gal4* genotype used as wild type. **A.** Survival of newly-eclosed adults exposed to starvation was tracked. Cmi KD animals survive significantly longer while Cmi OE animals die significantly sooner. (*N = 25 for each genotype; statistical significance measured by Log-rank Test*) **B.** TAG levels were assayed from representative cohorts exposed to starvation. Cmi KD animals depleted TAGs at a significantly lower rate than control. The depletion rate of Cmi OE animals cannot be compared to control, as the two populations begin starvation with different stored levels. (*N = 4 for each genotype; statistical significance measured by Student's T Test*)

Cmi Overexpression in the Fat Body Does Not Affect Triglyceride Depletion or Synthesis in the Adult

Depletion rate of TAGs during starvation is regulated by many factors, including by the current level of stored TAGs; the less TAGs available, the lower the rate of depletion [140]. Cmi OE and control adults exit metamorphosis with significantly different TAG levels, therefore the TAG depletion rate of Cmi OE animals compared to control cannot be accurately analyzed by the starvation beginning at this point. To account for this, adults of each genotype were collected after eclosion and aged four days until a homeostatic adult TAG level was achieved, which proved to be statistically similar among each genotype (**Fig. 20A**). After one day of starvation of these animals, TAG levels were analyzed. While Cmi KD animals once again demonstrated higher remaining TAG levels, Cmi OE TAGs were similar to control, suggesting that during adult starvation excess Cmi levels do not affect the depletion rate of TAGs. Additionally, while aged Cmi KD animals survived significantly longer than control, the starvation sensitivity of Cmi OE animals was abrogated (**Fig. 20B**). These results suggest that the increased lethality of starved Cmi OE animals, unlike the resistance of Cmi KD, is not due to altered depletion rate during starvation, but only TAG storage level at the onset.

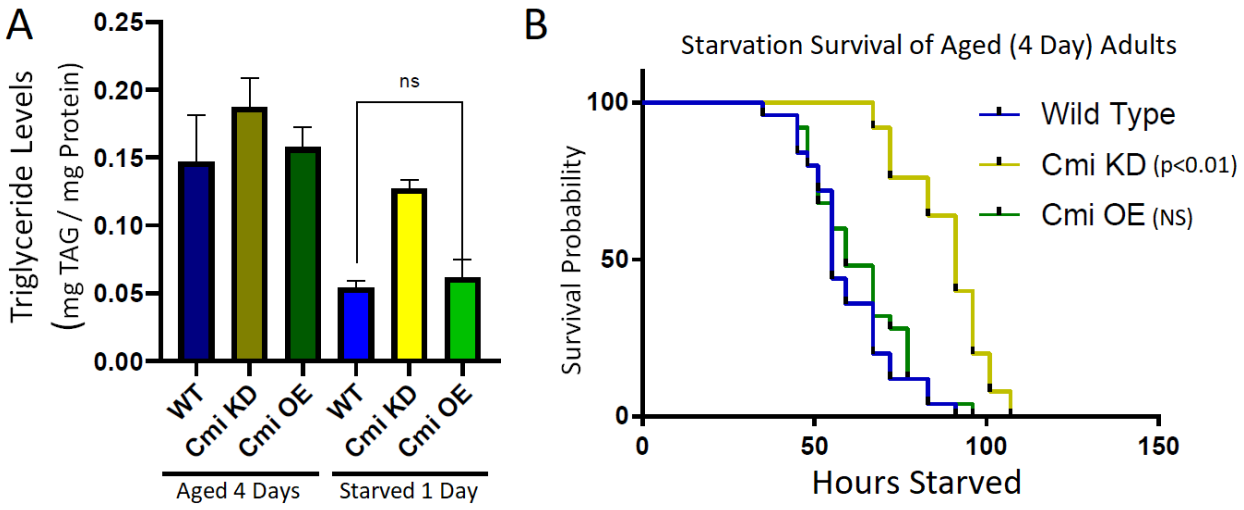

Figure 20. Cmi Overexpression in the Fat Body has No Effect on Triglyceride Depletion During Starvation. Cmi KD and Cmi OE constructs driven in the fat body using Lsp2-Gal4. Newly-eclosed adults were aged 4 days to reach a homeostatic adult TAG level and then starved for one day. *OregonR/Lsp2-Gal4* genotype used as wild type. **A.** TAG levels were assayed in representative cohorts from each timepoint. After aging four days, each genotype displayed similar TAG levels. After 1 day of starvation, depleted TAG stores of Cmi OE animals were similar to control. (*N = 4 for each genotype; statistical significance measured by Student's T Test, NS = not significant*) **B.** Adults aged 4 days were starved and survival tracked. Cmi KD animals survived significantly longer than control, yet Cmi OE animals died at a similar rate to control. (*N = 25 for each genotype; statistical significance measured by Log-rank Test, NS = not significant*)

Larval synthesis rate and storage level of TAGs is not sensitive to Cmi level in the fat body (**Fig. 16**). To determine if the same is true in adult fat body, adults were starved for one day post-eclosion and then transferred to nutrient rich medium, allowing them to feed and regain TAGs. After one day of starvation, Cmi OE and control adults had similar levels of stored TAGs remaining (**Fig. 21**). At both one and two days of refeeding, Cmi OE and control animals still displayed statistically similar TAG levels. These results suggest that excess Cmi levels in the fat body have no effect on the ability of adults to synthesize and store TAGs. Unfortunately, the TAG synthesis rate of Cmi KD adults cannot be compared to control, as synthesis rate (similar to

depletion rate) is partially regulated by current TAG level and the two genotypes begin refeeding at significantly different levels.

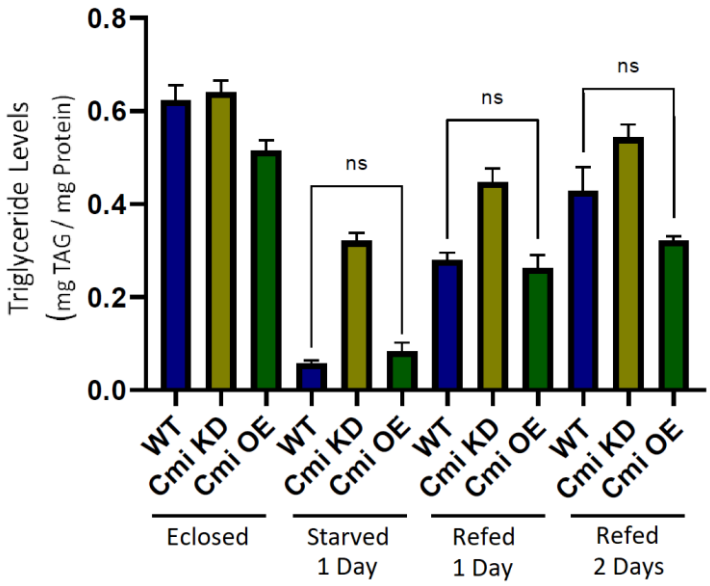

Figure 21. Cmi Overexpression in the Fat Body has no Effect on Triglyceride Replenishment during Refeeding. Cmi KD and Cmi OE constructs driven in the fat body using Lsp2-Gal4. Newly-eclosed adults were starved for one day and then refed for two days. TAG levels were assayed in representative cohorts from each timepoint. *OregonR/Lsp2-Gal4* genotype used as wild type. Cmi OE animals restored TAG levels at a similar rate to control. The TAG replenishment rate of Cmi KD animals cannot be compared to control, as the two populations begin refeeding with different stored levels. (*N = 4 for each genotype; statistical significance measured by Student's T Test, NS = not significant*)

Overall, these results suggest that the pupal and the adult fat body is sensitive to the level of Cmi during nutrient stress. Specifically, overexpression of Cmi increases the TAG depletion rate during metamorphosis and knockdown of Cmi decreases the TAG depletion rate during adult starvation. These suggest that the MLR complex serves a role to promote or enhance the animal's ability to mobilize and use stored TAGs during nutrient stress. However, differential sensitivity to Cmi levels in pupae versus adults suggests that the effects of MLR complex modulation may be through different mechanisms at different developmental stages.

Expression of Stress Response Genes is Sensitive to Cmi Levels in the Fat Body

If the MLR complex is necessary to regulate the fat body's ability to deplete TAG stores during nutrient stress, it would occur through transcriptional regulation. A number of potential direct or indirect regulatory targets may underlie the observed phenotypes. If the MLR complex is required for correct expression of the machinery that mobilizes TAG stores in adipocytes, such as lipases Bmm or Lip3 [129], then modulation of MLR activity would have a direct relationship to the depletion rate. The complex may also play an indirect role in regulating TAG depletion through regulation of hormone response or stress pathways. MLR complexes act as co-regulators of steroid hormone receptor transcription, including ecdysone receptor (EcR) in Drosophila [51]. Ecdysone hormone levels are increased during nutrient stress and EcR acts antagonistically towards insulin signaling in the fat body, promoting stress response and TAG mobilization [186–188]. The MLR complex may be necessary for promoting this nutrient stress role of EcR signaling in the fat body through direct regulation of EcR targets such as Ilp6 [132]. Additionally, activity of master stress response regulator Foxo in the fat body antagonizes pro-growth and feeding signals during nutrient stress and phenocopies the transcriptional reprogramming during starvation [135,154].

To perform an unbiased investigation of potential dysregulated regulatory targets of the MLR complex underlying the nutrient stress phenotypes, I prepared samples for RNA-seq analysis. The hormone ecdysone triggers systemic developmental transitions and transcriptional reprogramming during the animal's life cycle. To investigate how altered Cmi levels may effect reprogramming during such a transition, I chose to harvest tissue from two adjacent developmental stages: the "blue gut" (BG) larval stage occurs just prior to a major

ecdysone spike triggering pupariation and the commencement of metamorphosis, while the "clear gut" (CG) stage occurs immediately afterwards. Fat body tissue was isolated from control, Cmi KD, and Cmi OE cohorts at both BG and CG, poly-A RNA was isolated from these tissue samples, and RNA-seq was performed. Gene ontology (GO) and gene set enrichment analysis (GSEA) were performed on the RNA-seq datasets to identify gene categories significantly affected by knockdown or overexpression of Cmi.

Unexpectedly, lipid metabolic processes were not identified as significantly affected upon reduction or excess of Cmi. Instead, among the most significant GO and GSEA categories identified comparing Cmi KD or Cmi OE to control in both BG and CG stages were stress response groups (**Table 5**). These include antimicrobial/immune, heat shock, insecticide, and oxidative stress/hypoxia categories, comprising dozens of stress response genes that are similarly dysregulated upon modulation of Cmi in the fat body. Through manual analysis of these results, two notable patterns were discovered. Firstly, expression of master stress regulator Foxo's fat body targets (including *Ilp6*, *Thor(4E-BP)*, *Bmm*, and *Gnmt*) were upregulated upon Cmi KD and downregulated upon Cmi OE (**Fig. 22**), suggesting that the MLR complex is necessary for suppressing Foxo activity in the fat body.

Table 5. Stress Response Pathways are Significantly Dysregulated upon Knockdown or Overexpression of Cmi in the Fat Body. Gene ontology (GO) analysis was performed comparing gene expression profiles of blue gut larvae. **A.** Top four GO categories upregulated upon Cmi KD. **B.** Top four GO categories downregulated upon Cmi OE.

GO Groups Significantly **Upregulated** Upon **Cmi Knockdown**

Gene Ontology Group	p Value
Transmembrane Transport	2.00E-10
Oxidation-Reduction Process	4.80E-10
Heat Shock-Mediated Chromosome Puffing	7.90E-06
Response to Heat	1.00E-05

GO Groups Significantly **Downregulated** Upon **Cmi Overexpression**

Gene Ontology Group	p Value
Antibacterial Humoral Response	2.80E-10
Defense Response to Gram-Positive Bacterium	3.60E-10
Humoral Immune response	2.10E-08
Innate Immune Response	9.70E-08

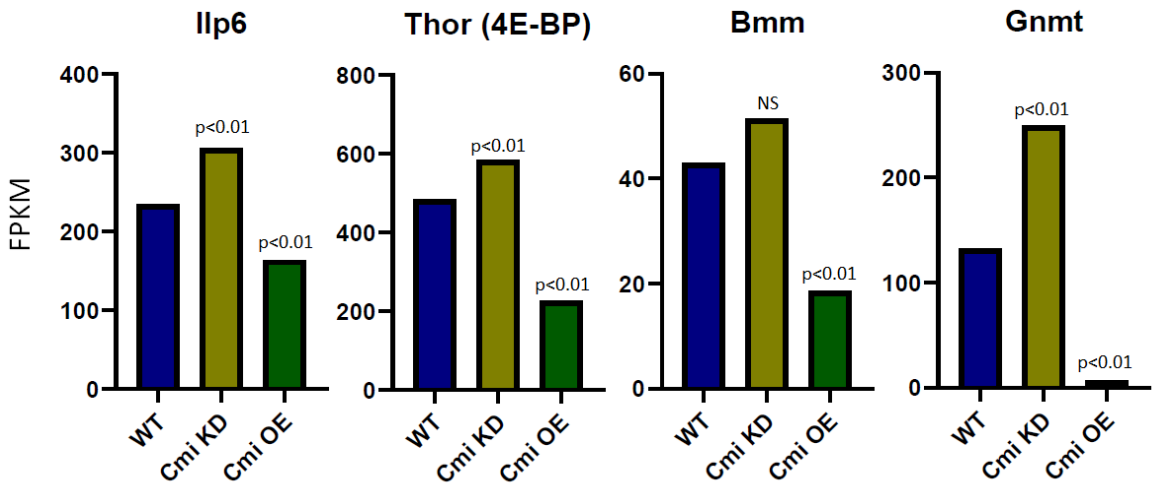

Figure 22. Cmi Fat Body Level Negatively Correlates with Expression of Foxo Targets. RNA-seq-derived FPKM of genes from W3L blue gut stage of each genotype are displayed. *OregonR/Lsp2-Gal4* genotype used as wild type. Foxo transcriptional targets in the fat body Thor, Ilp6, Bmm, and Gnmt are all upregulated upon Cmi KD and downregulated upon Cmi OE. (*Statistical significance determined by Novogene Genomics Services, NS = not significant*)

Secondly, a number of antimicrobial molecules demonstrate a similar pattern of dysregulation upon Cmi KD and OE (**Fig. 23**). Normally, the expression of these proteins is significantly upregulated during the reprogramming transition from BG to CG. However, upon Cmi KD, this relationship is reversed: expression is aberrantly high in the BG, but is downregulated upon transition to CG. Cmi OE animals demonstrate low BG expression that fails to properly upregulate in CG. The reversal upon Cmi KD is strikingly similar to effects of Cmi loss on EcR target genes that our lab has recently characterized [47]. The MLR complex plays roles in both readying EcR target genes for later activation as well as suppressing premature activation; the expression results of antimicrobial peptides suggest that the complex plays a similar role on innate immune genes in the fat body.

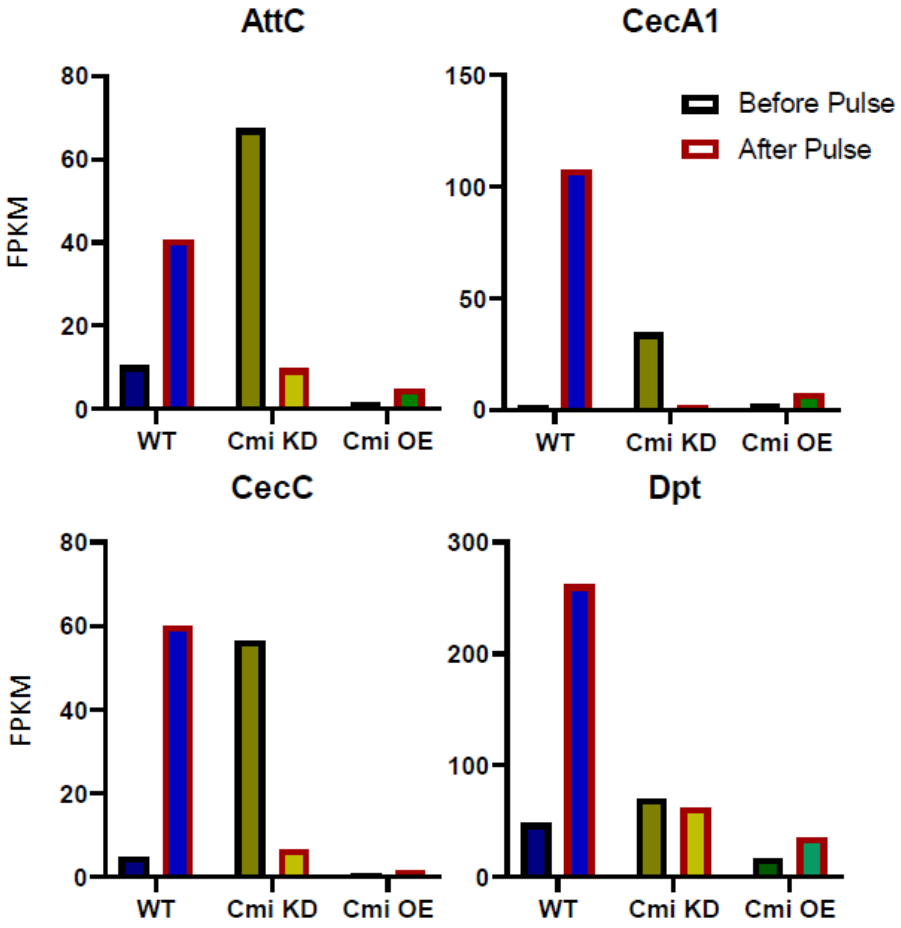

Figure 23. Antimicrobial Peptide Expression is Sensitive to Cmi Level in the Fat Body. RNA-seq-derived FPKM of genes from W3L blue gut (BG) and clear gut (CG) stages of each genotype are displayed. *OregonR/Lsp2-Gal4* genotype used as wild type. In wild type fat body, expression of antimicrobial peptides (AMPs) AttC, CecA1, CecC, and Dpt is upregulated upon transition from BG to CG stage. Upon Cmi KD, AMP expression is increased in the BG and decreased in the CG compared to control. Upon Cmi OE, AMP expression in decreased in both the BG and CG compared to control.

Starvation Survival is Sensitive to Fat Body Foxo Levels

Cellular metabolism and stress response are interwoven processes, particularly in the Drosophila fat body. Sensitivity of stress-reactive transcription to Cmi level suggests that the observed metabolic phenotypes may be indirect effects of dysregulated stress response; particularly suggestive is the apparent negative regulation of Foxo activity by the MLR complex.

Under starvation conditions, Foxo is translocated into the nucleus and transcriptional function is activated to reprogram in response to the stress, including promotion of TAG lipolysis through Bmm, regulation of body growth through Ilp6, and translational inhibition through Thor (4E-BP) [135,154]. To investigate if altered Foxo activity underlies the metabolic phenotypes, I used Lsp2-Gal4 to drive overexpression (Foxo OE) or knockdown (Foxo KD) of Foxo in the fat body and exposed the resulting newly-eclosed adults to starvation. Based on the inverse relationship between Cmi and Foxo activity suggested by the RNA-seq data, I anticipated that Foxo OE would mimic the effects of Cmi KD (starvation resistance) and that Foxo KD would mimic Cmi OE (starvation sensitivity). The results demonstrated that the ability of the animal to survive starvation was in fact sensitive to levels of fat body Foxo (**Fig. 24A**). Unexpectedly, however, Foxo KD provided a significant protective effect similar to Cmi KD, and Foxo OE significantly increased starvation susceptibility similar to Cmi OE. These results suggest that that the animal is similarly sensitive to Cmi and Foxo levels in the fat body during starvation, potentially through shared mechanisms. To test this, adults aged four days to attain a homeostatic adult TAG level were also exposed to starvation. Just as in Cmi OE animals, removal of the initial TAG differential abrogated the susceptibility of Foxo OE animal to starvation lethality (**Fig. 24B**), further supporting the concept that the MLR complex and Foxo interact mechanistically in regulating TAG depletion.

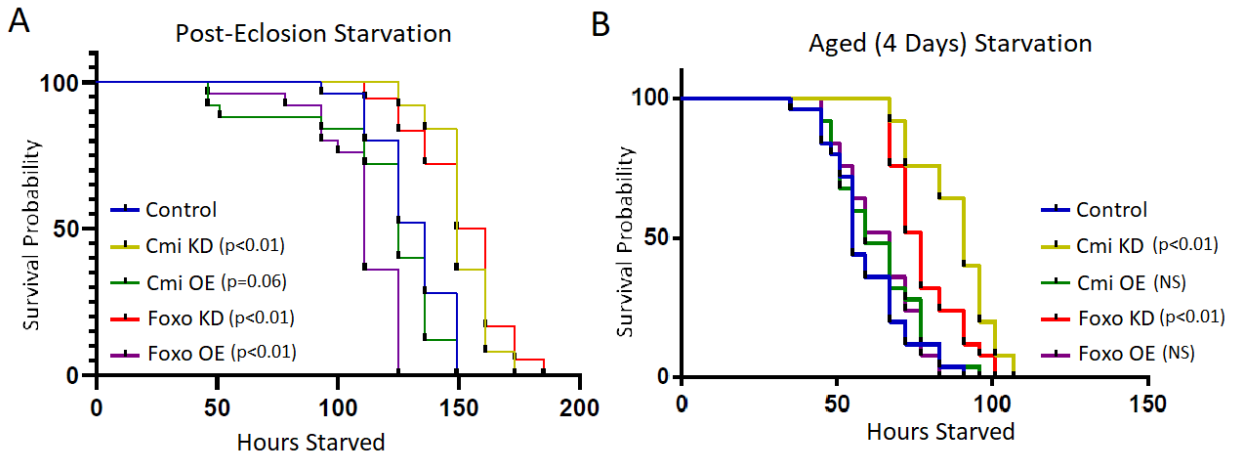

Figure 24. Starvation Survival is Sensitive to Foxo Level in the Fat Body. All genetic constructs driven in the fat body using Lsp2-Gal4. *OregonR/Lsp2-Gal4* genotype used as wild type. **A.** Survival of newly-eclosed adults exposed to starvation was tracked. Cmi KD and Foxo KD animals survive longer while Cmi OE and Foxo OE animals die sooner. **B.** Survival of four day-old adults exposed to starvation was tracked. Cmi KD and Foxo KD animals survive longer while Cmi OE and Foxo OE has no effect on survival. (*N = 25 for each genotype; statistical significance measured by Log-rank Test, NS = not significant*)

The MLR Complex is Required in the Fat Body for Oxidative Stress Response

As general stress response gene expression patterns in the fat body are dysregulated upon modulation of Cmi levels, I sought to test if Cmi KD or Cmi OE also affect the animal's ability to survive other stressors, such as oxidative stress. Foxo is activated upon exposure to oxidative stress and loss of Foxo reduces the animal's ability to respond to and survive it [135,153]; its role in the fat body specifically in responding to this stress, however, is less certain [189]. To investigate the potential roles of both the MLR complex and Foxo in the fat body's response to oxidative stress, I exposed adult animals knocking down or overexpressing either *Cmi* or *Foxo* in the fat body to either hydrogen peroxide (H_2O_2) though feeding. Either Cmi OE or Foxo OE reduced the animal's ability to survive oxidative stress while either Cmi KD or Foxo KD significantly their survival compared to control (**Fig. 25A**). These results demonstrate that both

the MLR complex and Foxo are required in the fat body for proper response to systemic oxidative stress, and suggest that the activities of both are somehow detrimental to survival. Interestingly, whereas the increased susceptibility of Cmi OE animals to starvation lethality is abrogated by aging the animals, the susceptibility to oxidative stress is not (**Fig 25B**). This suggests regulation of different mechanisms affecting survival in the different stress states.

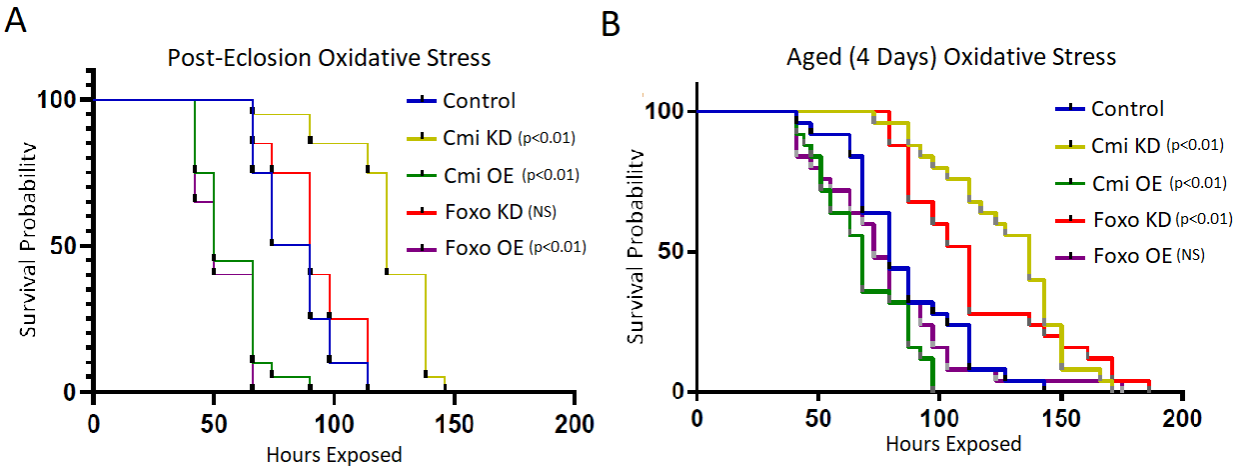

Figure 25. Oxidative Stress Survival is Sensitive to Cmi and to Foxo Levels in the Fat Body. All genetic constructs driven in the fat body using Lsp2-Gal4. *OregonR/Lsp2-Gal4* genotype used as wild type. **A.** Survival of newly-eclosed adults exposed to oxidative stress via H_2O_2 feeding was tracked. Cmi KD and Foxo KD animals survive longer while Cmi OE and Foxo OE animals die sooner. **B.** Survival of four day-old adults exposed to oxidative stress via H_2O_2 feeding was tracked. Cmi KD and Foxo KD animals survive longer while Cmi OE and Foxo OE animals die sooner. (*N = 25 for each genotype; statistical significance measured by Log-rank Test, NS = not significant*)

CHAPTER 5

DISCUSSION

The highly-conserved MLR COMPASS-like complexes are recruited to establish enhancer regions during development, and are thereby necessary for progressing the transcriptional activity of developmental signaling effectors, nuclear receptors, lineage determining factors, and other binding partners. The results described here advance this knowledge by further elucidating the roles of an MLR complex in two important contexts: differentiating cells in developing tissues, and reprogramming of a metabolic organ in response to stress states. Through my investigation into the MLR complex's regulation of the *bantam* miRNA during compound eye development, I have demonstrated for the first time that the complex plays roles in both positively and negatively regulating a single transcriptional target in the same tissue depending on cell fate. Using that same developmental system, I provide *in vivo* evidence of the necessity of the MLR complex to prepare enhancers for future activation, and its dispensability in maintaining enhancer function after activation, a relationship previously suggested through *in vitro* evidence. In the Drosophila fat body, I demonstrate that the function of adipocytes to mobilize and deplete TAG reserves is sensitive to MLR complex activity. I provide evidence suggesting that this is likely not a direct effect, but instead due to the requirement of the MLR complex for proper transcriptional reprogramming during stress

responses, an unanticipated but potentially significant role for the complex, and additionally demonstrate that response to oxidative stress also requires MLR complex activity. This work further elucidates the function of an epigenetic regulator critical for development and mutated in human disease.

Regulation of *bantam* by the MLR Complex is Required for Proper Adult Organ Formation

MLR Complexes are recognized as necessary co-regulators of many conserved developmental signaling pathways; therefore, it is unsurprising that reduction of MLR complex activity via knockdown of central subunits *Cmi* or *Trr* in imaginal tissues causes developmental defects. However, through detailed dissection of the resulting phenotypes and epistasis experiments, candidate binding partners and transcriptional targets can be identified, further clarifying how MLR complexes are used during cellular differentiation and organismal development. The work presented here takes advantage of *Drosophila melanogaster* as a genetic model to perform *in vivo* experiments investigating the effects of loss of MLR complex activity as discrete stages of development. The MLR complex is a necessary coactivator of Dpp, Hippo, and Notch signaling pathways, all of which converge on transcriptional regulation of the *bantam* miRNA; these results confirm *bantam* as a direct regulatory target of the complex during organ development. Through investigation of this single transcriptional target, my work further clarifies how MLR complexes orchestrate transcriptional regulation and fine-tune gene expression in developing animals.

Suppression of bantam Expression by the MLR Complex is Necessary for Proper Wing Formation

Our lab has previously described that wing formation is sensitive to levels of Cmi, and that resulting wing vein defects are due to positive regulation of Dpp signaling by the complex [11,52]. I demonstrate here that these same defects are sensitive to *bantam* levels: *bantam* reduction completely suppresses the phenotypic effects of Cmi knockdown and significantly enhances the effects of Cmi overexpression; *bantam* overexpression enhances the vein retraction of Cmi knockdown. The MLR complex is required to negatively regulate *bantam* expression in the developing wing, and a recent report has identified *bantam* as a negative regulator of Dpp signaling through translation inhibition of Mad [125]. Together, these data suggest a model in which the MLR complex controls Dpp signaling in the wing at two levels: positive regulation of Dpp signaling activity enhanced by negative regulation of *bantam* (**Fig. 26**). Dysregulation of *bantam* expression alone is not sufficient to result in the wing vein defects associated with Cmi modulation, therefore the phenotypes are caused primarily by altered Dpp transcription. These conclusions demonstrate how the MLR complex is critical for directly and indirectly regulating developmental signaling pathways at multiple stages, fine-tuning the transcriptional outputs necessary for proper organ formation.

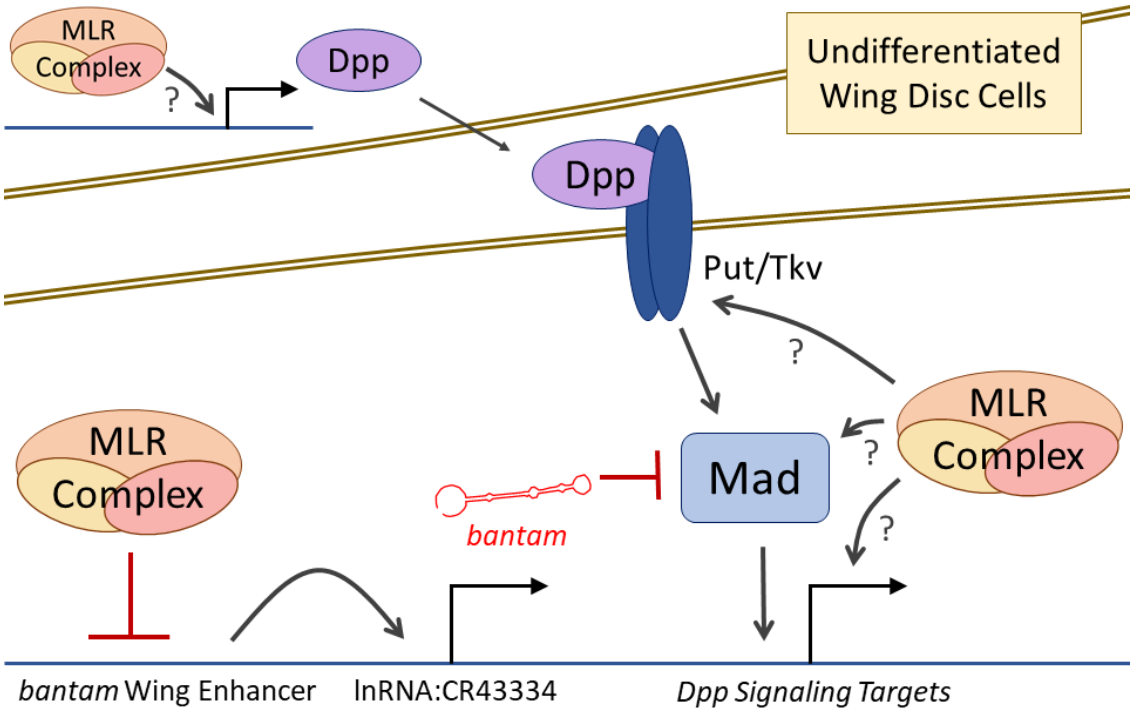

Figure 26. Model for MLR Complex Regulation of *bantam* and Dpp Signaling in the Wing Disc. We have previously demonstrated that the MLR complex is required to promote Dpp signaling activity in the wing disc (Chauhan 2013). This positive regulatory activity may occur at the level of transcription of Dpp itself, downstream Dpp machinery, or Dpp target genes. I propose a parallel second mechanism: suppression of the *bantam* wing enhancer, leading to downregulation of the miRNA *bantam* and reducing its ability to inhibit the translation of Dpp effector Mad.

Effects of MLR Subunit Knockdown on Eye Formation

Unlike the effects of *Cmi* knockdown or overexpression in the wing, the results of *Cmi* or *Trr* knockdown in the eye are complex are likely due to multiple dysregulated developmental signaling pathways. What I describe as a “rough and shrunken eye” phenotype is the result of several combined effects. Firstly, the “roughness” is due to disorganized ommatidia of the compound eye. SEM photography revealed that these pattern disruptions are due to multiple effects: lens fusion of neighboring ommatidia, altered ommatidial sizes, variation in ommatidial

border length and neighboring units, and multiple bristle defects including duplication, loss, and mispatterning events. Precise patterning during ommatidial development relies on proper differentiation of all involved elements; in other words, one altered event, such as cell loss/duplication/fate change may be causal to other events. Therefore, I cannot determine from this data the defects that are directly caused by loss of MLR complex activity that lead to the broad mispatterning phenotype. Secondly, the "shrunken" eye could be caused by loss of tissue or the result of changes in cell fate during organ formation. While my data reveal that *Cmi*/*Trr* knockdown induces apoptosis in undifferentiated eye cells, I also demonstrate that suppressing cell death does not also rescue eye size. Further evidence that reduced eye size is not primarily due to cell loss arises from the fact that animals with extremely shrunken eyes do not also exhibit correspondingly shrunken heads, but instead have increased head capsule area to compensate for eye loss. These results suggest that loss of MLR complex activity affects cell fate choice during head formation, favoring head capsule epidermal development over compound eye formation. In the eye disc, Wg signaling (a form of Wnt signaling) is required at the tissue margins to suppress eye development and allow for head capsule formation [190]. The KMT2D MLR complex has been shown to be necessary for the transcriptional activity of Wnt effector PITX [54], suggesting that loss of MLR complex function in the eye disc may dysregulate Wnt signaling and thereby disrupt eye/head capsule fate balance during development.

The sensitivity of the adult eye phenotype to *bantam* levels suggests that the phenotype is caused by dysregulation of developmental signaling pathway(s) regulated by *bantam*. The fact that *bantam* overexpression alone phenocopies the rough and shrunken eyes goes further to imply that the defects in *Cmi*/*Trr* knockdown eyes may be the direct result of increased

bantam levels. If so, this would most likely originate in the proneuronal ommatidial cells, as I determined that *Cmi*/*Trr* knockdown upregulates *bantam* in this population.

In addition to *bantam* overexpression, expression of caspase inhibitor p35 alone results in phenotypically similar rough and shrunken adult eyes. These data suggest that the defects are the result of inhibition of apoptosis rather that dysregulated developmental signaling. Precise apoptotic pruning of cells is necessary for proper ommatidial patterning; suppression of this leads to excess cells and a disrupted compound eye lattice. In addition, while caspases are best characterized as promoters of apoptotic cellular disassembly, many have non-apoptotic functions including regulation of differentiation and cell fate [191–193]. It is therefore possible that inhibition of apoptosis deleteriously effects the ability of the eye disc to properly develop into the compound eye.

Disparity Between Cmi and Trr Knockdown in the Eye. *Ey-gal4*-driven knockdown of *Cmi* and of *Trr* results in phenotypes with significantly different levels of penetrance and expressivity. Specifically, the Trr KD phenotype is completely penetrant with all eyes categorized as at least “completely rough and shrunken”, while the Cmi KD phenotype is approximately 75% penetrant with eyes varying in size as well as severity of roughness. This disparity may be due to multiple factors, the simplest possibility being variation in knockdown strength. We have verified both *Cmi-IR* and *Trr-IR* RNAi constructs in multiple contexts, including qualitative analysis in the eye disc through immunofluorescence staining. Unfortunately, quantitative biochemical measurement of knockdown efficiency in the eye disc is impossible, due to both the heterogenous nature of the tissue and specificity of *Ey-gal4* expression. However, measurements and observations in another organ system, the wing, has

demonstrated that our *Trr-IR* construct has greater knockdown efficiency than our *Cmi-IR*, which often necessitates the overexpression of *Dicer2* to enhance the knockdown efficiency. Another possible explanation for the difference in phenotypic severity involves the different effects of Cmi and Trr loss on the MLR complex. Loss of Trr reduces the stability of the complex and its other subunits, preventing formation. On the other hand, loss of Cmi allows the complex to form and bind to chromatin, but prevents methyltransferase and recruitment of p300/CBP [47]. This presents the possibility that MLR complexes lacking Cmi retain unidentified regulatory function requiring localization to target enhancers, leading to more severe effects when the complex cannot form and bind due to Trr loss. Importantly, knockdown of either *Cmi* or *Trr* does not result in different phenotypes, but rather different levels of severity of the same phenotype. This suggests that the disparity is not due to the dysregulation of different targets, but instead the intensity of that dysregulation.

The MLR Complex Directly Regulates Tissue-Specific *bantam* Enhancers

The results described here demonstrate that the MLR complex localizes to tissue-specific *bantam* enhancers during imaginal disc development, is necessary for regulating the activity of those enhancers, and is consequently required for proper *bantam* expression in the wing and eye imaginal discs. Together, these data establish the *bantam* miRNA as a direct regulatory target of the complex. While the exact binding partners that recruit the MLR complex to the *bantam* locus are not investigated here, the most likely candidates are those factors that have already been determined to require MLR complex activity as well as regulate *bantam* expression: Yki, Mad, and Notch. Yki binds to and regulates the wing and eye enhancer regions in conjunction with Sd (specific to wing enhancer) or Hth (specific to eye enhancer) [111].

The MLR Complex Negatively Regulates the bantam Wing Enhancer

The MLR complex is necessary for suppressing activity of the *bantam* wing enhancer and negatively regulating *bantam* transcription in the wing disc. Therefore, the activity is unlikely to occur through Yki/Sd, which positively regulate activity of this enhancer region. Direct negative regulation of targets by the complex is relatively uncharted territory; it has only been previously described once before, in a report by our research group demonstrating repression of hormone response elements [47]. In that investigation we suggest that the MLR complex plays a critical bookmarking-like role, remaining at primed enhancer elements prior to activation and preventing premature activation and initiation of transcription. The complex potentially plays a similar role at the wing enhancer. Another possibility is that the complex is somehow required for the activity of a repressor of *bantam*, such as Brk [122]. However, as repressive factors such as Brk function through recruitment of corepressors [194], it is difficult to speculate as to the involvement of a complex whose known functions are all associated with enhancer activation (H3K4me1 deposition, H3K27 demethylation, and p300/CBP recruitment).

The MLR Complex is Necessary for Proper Patterning of the bantam Eye Enhancer

Regulation of the *bantam* eye enhancer by the MLR complex is less straightforward than in the wing enhancer. Rather than a clear increase or decrease in activity upon knockdown of *Cmi*/*trr*, the eye enhancer displays disrupted patterning consisting of sporadic loss of activity in addition to ectopic activation. Also dissimilar to the wing, dysregulation of the *bantam* eye enhancer does not directly translate to a reciprocal alteration of *bantam* expression in affected cells. This specific enhancer region is only active in the very anterior margin of the eye disc within undifferentiated cells, and depletion of MLR activity has no effect on *bantam* levels in

that tissue. These results suggest that the MLR complex is not required to simply promote or suppress activation of the eye enhancer, but rather plays a role in spatiotemporal control of activation. This is consistent with our report demonstrating that MLR complexes have bookmarking-like capabilities that stimulate rapid enhancer activation under proper conditions and silence activation otherwise [47]. This interpretation would suggest a model under which the MLR complex is recruited to establish the *bantam* eye enhancer and other cis-regulatory regions early during imaginal disc development and remains to ensure appropriate activation (**Fig. 27**). Thereby reduction in complex activity results in two effects: failure to fully activate the enhancer in cells stimulated to do so, and failure to fully suppress the enhancer in cells less stimulated.

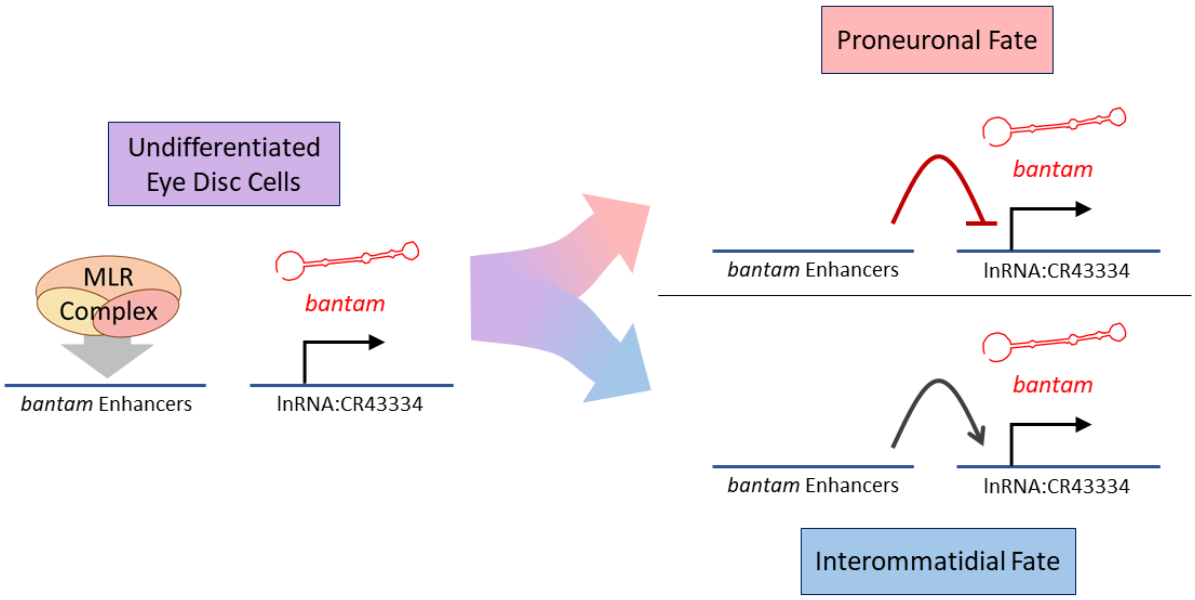

Figure 27. Model for MLR Complex Regulation of *bantam* in the Differentiating Eye Disc. In undifferentiated eye disc cells, the MLR complex is necessary for establishing as-yet unidentified *bantam* enhancers that will become active upon differentiation. Once differentiation commences and cell fate is chosen, these enhancers have different regulatory activity on *bantam* expression depending on that lineage decision. In proneuronal cells these are responsible for suppressing *bantam* transcription; in interommatidial cells these are responsible for promoting *bantam* transcription.

The MLR Complex is Required is Undifferentiated Eye Cells for Proper *bantam* Expression During Subsequent Differentiation

The Drosophila eye disc is a unique and valuable model when investigating developmental gene regulation, as it contains cells at various states of differentiation, from undifferentiated multipotent cells to those committed to a specific lineage and organizing into compound eye units. While the MLR complex is required to regulate the activity of the *bantam* eye enhancer in undifferentiated cells, depletion of complex activity has no effect on *bantam* transcription until differentiation commences and cell fate has been chosen. Experiments knocking down *Cmi*/*Trr* is specific sections of the developing eye revealed that the MLR

complex is necessary in undifferentiated tissue for proper *bantam* expression in differentiating eye cells, yet is dispensable once differentiation has begun. These results concur with the regulatory model described above: the MLR complex is recruited to and establishes *bantam*-specific enhancers in undifferentiated eye tissue without affecting *bantam* transcription in those cells. Upon cell fate determination during differentiation, those established enhancer regions regulate *bantam* expression (**Fig. 27**). If the MLR complex is not present in the undifferentiated cells, proper expression in differentiating cells is deleteriously affected. This model echoes *in vitro* data demonstrating that the mammalian Kmt2D MLR complex is required for transcriptional reprogramming during differentiation, but unnecessary for maintaining expression patterns once adopted [21]. The results described here not only verify these observations in an *in vivo* developmental model, but demonstrate that the priming regulatory activity by the complex can occur several cell generations prior to its transcriptional effects, as undifferentiated eye cells undergo at least two rounds of mitosis before lineage determination [101,103]. This suggests that the MLR complex's enhancer establishing role is maintained past nuclear division and mitosis.

The eye enhancer represents only a single regulatory input on *bantam* expression and is likely not the main transcriptional controller in undifferentiated eye tissue. Our ChIP-seq results demonstrate that Cmi is enriched throughout the *bantam* locus and may bind to as-yet unidentified enhancer regions. The fact that *bantam* levels are unaffected by *Cmi*/*trr* knockdown in this region should not be interpreted to mean that the MLR complex plays no role in regulation of the miRNA in undifferentiated eye cells. Consistent with my model of *bantam* regulation in differentiating ommatidia, I favor the hypothesis that the establishment

of regulatory elements controlling *bantam* in these cells occurs very early during or prior to imaginal disc formation, and that the complex is required at that developmental timepoint. Ey-Gal4 is active only after eye-specific fate has been determined, and our RNAi would deplete the MLR complex only after enhancer establishment. The MLR complex is dispensable for maintaining enhancer activity once induced, therefore I would not expect Ey-Gal4-driven knockdown to affect *bantam* levels in undifferentiated eye cells.

The MLR Complex is Required for Regulating *bantam* Expression Differentially Depending on the Context of Cell Fate

The MLR complex is not only necessary for regulating *bantam* transcription in differentiating ommatidia, but it does so through either promotion or suppression of *bantam* expression, depending on the context of cell fate. The requirement of the complex to either positively or negatively regulate a single transcriptional target in the same tissue is a novel observation consequential to proper development. This regulatory "decision" is likely not inherent to function of the MLR complex itself, but rather depends on when, where, and by what factors it is recruited to *bantam* regulatory regions. Multiple regulatory inputs are required to orchestrate proper developmental expression of the *bantam* miRNA [111,195], and these results suggest that the MLR complex plays a critical role in translating these inputs into regulatory decisions.

The MLR Complex is Necessary in Undifferentiated Eye Cells to Promote Survival

The MLR complex is not only required to establish enhancer regions in undifferentiated imaginal tissue for later regulatory activity, but also has an unanticipated role in protecting these cells against apoptotic cell death. Knockdown of *Cmi*/*Trr* in the wing disc results in a

widespread increase in caspase activation, while knockdown in the eye disc causes caspase activation and apoptotic death in a concentrated region of the organ: the anterior section of the dorsal-ventral midline. This particular location in the eye disc in conjunction with the phenotype's sensitivity to *bantam* levels suggests involvement of the Notch signaling pathway, which functions in that area to promote survival of undifferentiated eye cells and is involved in a positive regulatory feedback loop with *bantam* [105,126]. This hypothesis is further supported by the fact Cmi/Trr KD reduces activity of a Notch response element reporter in that same region of the eye disc. Together, these data suggest a model in which the MLR complex is necessary for the promotion of cell survival by Notch signaling in undifferentiated eye cells, most likely through positive regulation of eye-specific transcription factor Eyg (**Fig. 28A**).

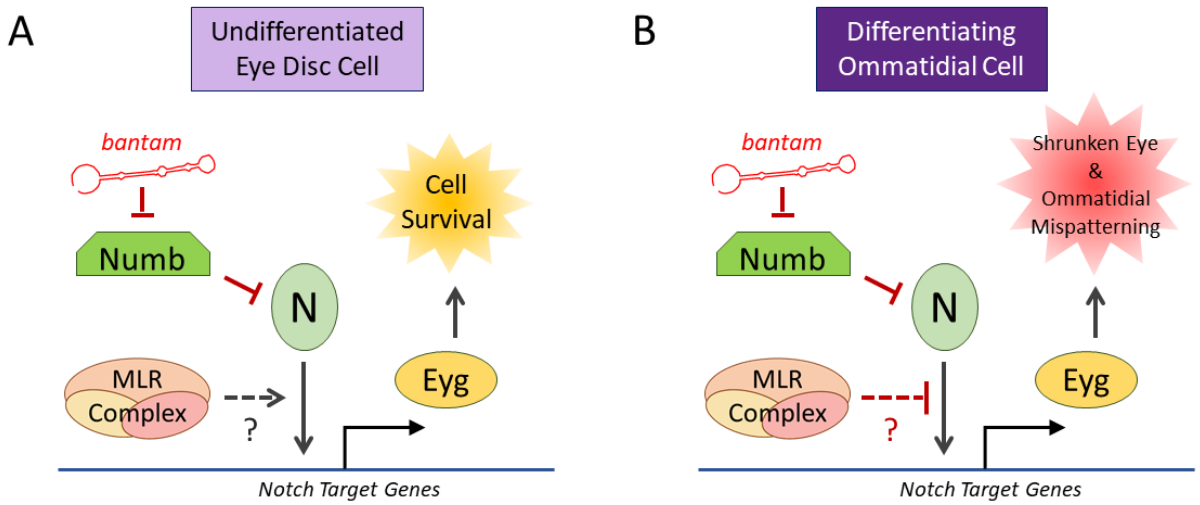

Figure 28. Model for MLR Complex Regulation of Notch Signaling in the Developing Eye. Notch signaling is required in undifferentiated eye disc tissue to promote survival; my results suggest that excess Notch signaling activity in the differentiating compound eye leads to malformations including shrunken eye size and ommatidial mispatterning. The MLR complex promotes Notch signaling activity in the former case and antagonizes in the latter. The exact mechanisms of regulation, including at what level the Notch signaling pathway is regulated, are unknown.

Requirement for MLR Complex During Eye Development Likely through Notch Signaling

While the aforementioned data suggests that the MLR complex is required to positively regulate Notch signaling in undifferentiated eye cells, genetic interaction evidence suggests the opposite during subsequent compound eye development. The rough and shrunken eye phenotype is suppressed by reduced Notch activity while it is enhanced by increased Notch activity as well as overexpression of Notch target Eyg, demonstrating an inverse relationship between MLR complex function and Notch signaling during eye development. This observation in conjunction with the fact that overactivation of Notch signaling alone phenocopies the effects of Cmi/Trr KD suggests that the adult malformations are caused by aberrantly high or ectopic Notch signaling during the development of the compound eye (**Fig. 28B**). This

interpretation provides a new perspective on the *bantam* interaction data; as *bantam* is an indirect promoter of Notch activity, the *Cmi/Trr* knockdown phenotype's sensitivity to *bantam* levels may be due to indirect modulation of Notch signaling.

Just as proper Notch signaling is necessary for survival in the undifferentiated eye [105], it is also required within differentiating ommatidia for proper cell fate determination [196]. This suggests that improper Notch target regulation upon loss of MLR complex activity leads to a rough and shrunken eye through dysregulation of cell fate determination, disrupting ommatidial pattern formation and potentially affecting the balance between eye and head capsule size. Notch functions through lateral induction and inhibition during tissue patterning, resulting in lattice networks determining cell fate. This means that even if the MLR complex is required for proper Notch signaling activity only within cells of a certain fate, this may have cascading regulatory effects on neighboring cells, potentially explaining how the patterning of multiple cell types of the adult compound eye are affected by loss of MLR complex activity.

My conclusions assert that the two phenotypic effects of Cmi/Trr KD in the eye disc (apoptosis of undifferentiated cells and malformation of the adult organ) are not only mechanistically separate, but in fact caused by altering Notch signaling in opposite directions. Just as the MLR complex is necessary for regulating the transcription of the *bantam* miRNA in different directions depending on cell fate, these data suggest that its effects on Notch activity depend on developmental stage. Previous evidence of the regulation of Notch signaling by MLR complexes appears conflicting: an MLR complex competes against NCoR to permit an active chromatin environment at Notch targets [57], yet there are multiple cases of MLR complex activity resulting in downregulation of Notch machinery and reduced Notch signaling function

[55,56,58]. Each of these reports focuses on a different developmental context and my model suggests that the MLR complex functions to fine-tune Notch signaling intensity, potentially at multiple levels of direct or indirect regulation, depending on developmental context and the particular requirements of the tissue.

The MLR Complex Positively Regulates Triglyceride Depletion in the Fat Body

While MLR complexes are well-characterized as required for proper transcriptional reprogramming in response to developmental signaling factors during differentiation and development, they are also integral for reprogramming in response to regulatory cues in other contexts. As co-regulators of FXR and p53 targets, MLR complexes are required for reprogramming responses to homeostatic maintenance cues in terminally differentiated cells [60,61,85,86]. My results demonstrate that an MLR complex is also necessary for regulating the depletion rate of stored TAG for energy during nutrient stress, a function critical for survival that must be precisely regulated.

Evidence from two stages of nutrient stress requiring energy from TAG stores, metamorphosis and adult starvation, suggest that the MLR complex in the fat body plays a role in suppressing TAG depletion. However, these sensitivities appear to be stage-specific: Cmi OE significantly increases TAG depletion rate during metamorphosis but not during starvation; Cmi KD inhibits TAG depletion rate during starvation but has no effect during metamorphosis. Importantly, while the non-feeding periods of metamorphosis and adult starvation both promote depletion of TAGs for energy, they involve very different reprogramming events. Metamorphosis is a proactive developmental period of nutrient stress during which time organismal metabolism is restructured in concert with the radical reorganization process of the

body. Starvation is a reactive period of nutrient stress involving temporary shifts in metabolism poised for return to homeostasis upon feeding stimuli. Given these, it's likely that the MLR complex either regulates fat metabolism through different transcriptional targets in each instance, or through the same transcriptional target(s) regulated by different stimuli during the two developmental periods.

The MLR Complex is Necessary for Regulating Stress Response in the Fat Body

The RNA-seq data collected from larval fat bodies suggest that among the most significate transcriptional effects of Cmi modulation is dysregulation of stress response genes, including those involved in antimicrobial, oxidative, and thermal stress. Hydrogen peroxide survival assays demonstrated that the ability of the animal to survive oxidative stress is indirectly related to the level of Cmi in the fat body.

A likely mechanism of the MLR complex's impact on stress response is through the regulation of Foxo activity. Many Foxo target genes are upregulated upon Cmi KD and downregulated upon Cmi OE, suggesting that the MLR complex has a role in suppressing Foxo activity. This is challenged by the fact that the ability to survive oxidative stress indirectly correlates with Foxo level just as it does with Cmi level in the fat body, suggesting a positive mechanistic relationship between the two. While it is difficult to incorporate these two seemingly conflicting observations, it's important to note that the expression data and phenotypic data examine different developmental timepoints (late larval and adult, respectively). As determined above, the MLR complex may interact with the same transcription factors and regulate the same targets in opposite directions depending on developmental context.

AMPs expressed by the fat body in response to microbial invasion and upregulated upon metamorphosis are dysregulated upon Cmi KD in a pattern similar to ecdysone-response genes [47], suggesting that the MLR complex positively regulates the transcription of these genes and is required to suppress their premature activation. These data suggest that the MLR complex acts as a co-regulator of one or more of the Drosophila NF-κB-like effectors Relish, Dl, and Dif.

The MLR Complex May Indirectly Affect TAG Depletion through Regulation of Stress Response Genes

Metabolism and stress response are intrinsically linked and regulation of one impacts the other. Under nutrient stress, Foxo activity promotes TAG depletion rate through positive regulation of Bmm expression, whereas activity of Relish suppresses Bmm transcription; balanced regulatory activity between the two promotes survival [168]. Based on my current evidence, I hypothesize that the MLR complex negatively affects TAG depletion during non-feeding periods through transcriptional regulation of TAG lipase Bmm through interaction with Foxo and/or Relish (**Fig. 29**). This implies positive or negative regulation of Foxo and/or Relish transcriptional activity, respectively.

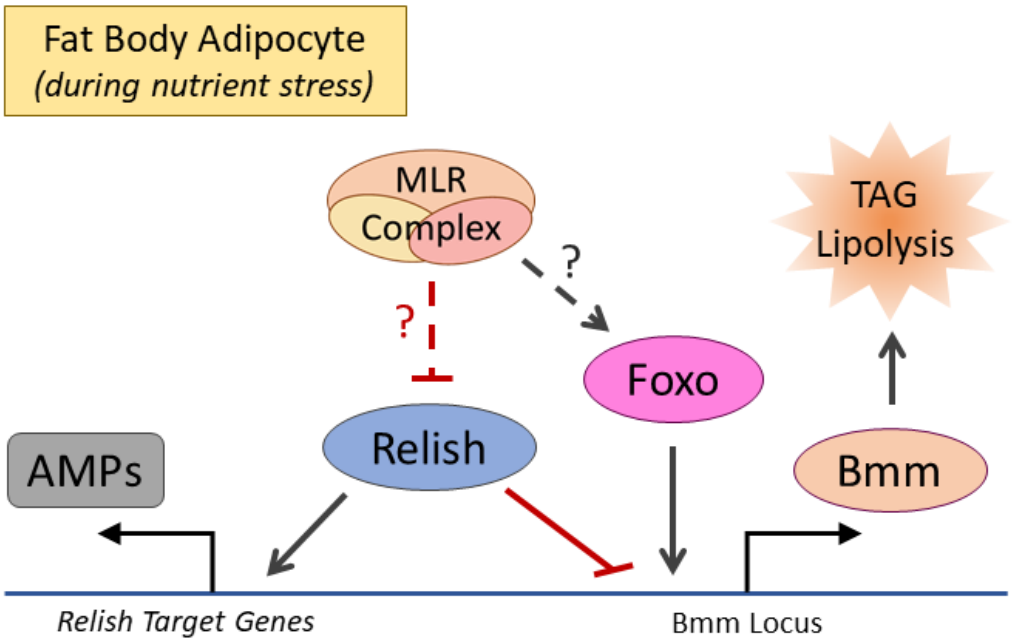

Figure 29. Model for MLR Complex Regulation of Foxo and/or Relish Activity in the Fat Body. My results suggest that MLR complex activity promotes TAG depletion during nutrient stress and is required to regulate targets of stress response effectors Foxo and Relish. TAG lipolysis is regulated by Bmm transcription, which is promoted by Foxo and inhibited by Relish. I propose that, in times of nutrient stress, the MLR complex is required to positively regulate Foxo activity and/or negatively regulate Relish activity, thereby controlling the rate of TAG depletion. The exact mechanisms of regulation, including at what level the Notch signaling pathway is regulated, are unknown.

The RNA-seq data demonstrates that multiple Foxo targets, including Bmm, are upregulated upon Cmi KD and downregulated by Cmi OE in larval fat body. Rather than interpreting this simply as negative regulation by the MLR complex, it may be another example of the MLR complex repressing premature gene activation. Under this interpretation, loss of MLR activity would cause an early upregulation of Foxo targets (as is seen in the late larva) followed by a later inability to stimulate those same targets (causing the Cmi/Foxo sensitivity in stress lethality).

The dysregulation pattern of the AMPs upon Cmi modulation in the fat body clearly suggests co-regulatory activity with one or more NF-κB analogues, including Relish. If this regulatory relationship is conserved at other Relish targets, the MLR complex may also be responsible for fine-tuning Bmm expression in response to lack of feeding. This would suggest that the MLR complex somehow antagonizes this repressive activity of Relish; as the negative regulatory role of Relish in this context is not yet mechanistically understood, it remains possible that the complex plays an undefined regulatory role.

The MLR complex interacting with and regulating the targets of either Foxo, Relish, or both would explain many of the phenotypes and expression patterns observed. My current evidence cannot distinguish between these possibilities.

CHAPTER 6

IMPACTS AND FUTURE DIRECTIONS

The MLR Complex is Required in Undifferentiated Cells for Proper Transcriptional Regulation During Differentiation

MLR complexes are dispensable for maintaining the expression of genes once activated, but are required for the reprogramming that occurs during differentiation, potentially through a role in enhancer poising [21,47]. While previously demonstrated *in vitro*, the timescale of this activity was unknown. My results confirm the *in vitro* data and go further to demonstrate that the enhancer establishment and poising activity of MLR complexes may occur multiple cell generations prior to the prepared-for reprogramming event. They suggest that enhancer establishment takes place in multipotent undifferentiated eye cells in preparation for reprogramming of *bantam* transcription once ommatidial development begins, and that the MLR complex is required for this establishment.

Intriguingly, the effects of loss of MLR activity on *bantam* expression are cell type-specific: upregulation in interommatidial cells and downregulation in proneuronal cells. This may be due to either direct or indirect regulatory activity by the MLR complex. If the activity is direct, then this can be interpreted in two ways: either the MLR complex regulates separate cell fate-specific enhancers and depletion affects these elements differently, or failure to establish the same enhancers has different effects based on cell fate. To continue investigating this

function, these enhancers must be identified. While chromatin-capture techniques could theoretically provide candidate regulatory sequences that contact the *bantam* promoter at these developmental timepoints, the separation and purification of eye disc cells according to cell fate is technically challenging. Instead, a wider range of previously-identified regulatory DNA surrounding the *bantam* locus can be fragmented into reporter lines, similar to enhancer trapping. These would be individually assayed for cell type-specific activity and sensitivity to MLR depletion, tracking these throughout eye development for a full time-scale of regulatory activity. Candidate sequences would then be interrogated for transcription factors binding based on consensus binding sequence identification and ChIP-seq data, identifying likely binding partners necessary for recruiting the MLR complex.

If no such MLR complex-dependent enhancer regions are identified, this would suggest indirect regulatory activity by the complex. This would likely be the result of upstream regulation of one or more developmental signaling pathways that drive *bantam* expression, including Hippo, Dpp, and Notch. Initial genetic interaction experiments modulating different components of these pathways would be performed to both identify candidate pathway(s) and to suggest at what level the MLR complex is required for proper regulatory activity.

It is also possible that the MLR complex has direct regulatory activity on *bantam* expression in one cell fate and indirect activity in the other. This includes the prospect that *bantam* transcription in one cell indirectly regulates *bantam* expression in the neighboring cell of a different fate, such as through the dysregulation of juxtacrine Notch signaling. Such a relationship would be technically difficult to identify, but suggestive evidence would include sensitivity within one cell fate and not the other during genetic interaction experiments.

MLR Complex Regulation of *bantam* Expression is Necessary for Proper Organ Formation

As a critical regulator of multiple developmental signaling pathways, the requirement for the MLR complex during organ formation is expected. The data presented here demonstrates that proper regulation of *bantam* expression by the complex is necessary for accurate tissue patterning, but does not definitively identify the target and mechanisms downstream of *bantam* that ensure proper organ development. In the wing, as discussed, the likely inhibitory target is Mad, modulating Dpp signaling for precise vein patterning; MLR activity thereby promotes Dpp signaling at two levels: positive regulation of Dpp expression and negative regulation of *bantam*. This would be confirmed in further experiments by assaying Mad levels by immunofluorescence upon modulation of *bantam*, and genetic interaction experiments between Dpp signaling components (Tkv, Mad) and *bantam*. The rough and shrunken eye associated with loss of MLR activity or increase in *bantam* activity is likely the result of multiple dysregulated developmental signaling pathways. Based on my data, I proposed that alteration of Notch signaling may play a major role in these effects. Previous investigations have claimed that MLR complexes suppress Notch signaling during organ development by negatively regulating the expression of Notch co-regulator RBPJ/Su(h) or Notch itself [55,56,58]. To investigate if these mechanisms are conserved here, differentiating eye cells would be stained for Notch or Rbpj to determine if their expression is sensitive to *Cmi*/*trr* knockdown. As a regulatory target of *bantam* (and therefore potential indirect regulatory target of the MLR complex), the expression and localization of required Notch inhibitor Numb would also be assayed via immunostaining. Numb functions to suppress Notch signaling activity at multiple levels: suppression of Notch cleavage at the cell membrane, sequestration of cleaved

Notch from the nucleus, and repression of Notch receptor recycling. If dysregulation of Numb expression is found to be causal to the rough and shrunken phenotypes, rescue experiments would be designed to intervene at multiple stages of Notch activation to determine at which level Numb is regulating Notch. Genetic interaction experiments would also continue, testing other components and downstream targets of Notch signaling in addition to other candidate regulatory pathways, such as Wnt signaling.

Previous reports detailing the effects of *bantam* overexpression in the eye disc describe different and opposite phenotypes than I have documented here. However, rather than conflicting with my results, these data taken together inform the functional roles of *bantam* at different developmental stages. When *bantam* is overexpressed solely within the differentiating ommatidial cells posterior to the morphogenetic furrow (via GMR-Gal4), the adult eyes are larger compared to control and display a roughness due to excess interommatidial cells [120,121]. My examples of *bantam* overexpression throughout the entire eye pouch (via Ey-Gal4) result in a similar roughness phenotype but also a decrease in eye size. Taken together, two conclusions can be made from these data. Firstly, that the size difference is due to *bantam* level in the undifferentiated eye cells, and that excess *bantam* in these cells promotes a small compound eye. The mechanisms resulting in size difference in either direction are unknown, although developmental pathways active in eye development and regulated by *bantam* (ie. Hippo, Dpp/TGF-β, and Notch) are likely candidates. Secondly, these data suggest that the roughness phenotype caused by additional interommatidial cells is due to excess *bantam* in differentiating ommatidia. An obvious assumption is that excess anti-apoptotic *bantam* in these interommatidial cells is blocking apoptotic pruning, resulting in extra cells.

However, Cmi/Trr KD mimics this phenotype and is associated with *bantam* downregulation in interommatidial cells and upregulation in proneuronal cells, suggesting that it is the aberrantly high *bantam* levels in proneuronal cells that is causing this effect. Testing this hypothesis would require modulation of *bantam* levels specifically within proneuronal cells, potentially using Elav-Gal4 or a similar transcriptional driver.

The MLR Complex Promotes Cell Survival in Undifferentiated Tissue

Many *in vitro* reports have determined that MLR complex activity is necessary in multipotent cells for differentiation capability, but not for maintaining growth and survival [21,31,42]. However, my *in vivo* developmental data demonstrates that the knockdown of MLR complex subunits in undifferentiated imaginal disc tissue results in increased apoptosis. Both the wing and anterior eye exhibit a sporadic increase of apoptotic cells upon loss of MLR complex activity, but the eye additionally demonstrates a significant concentration of programmed cell death on the dorsal-ventral midline, suggesting two different mechanisms requiring the MLR complex to promote cell survival in developing tissues. The sporadic, cell autonomous apoptotic effect suggest a general role in protecting against cell death. Multiple mechanisms may underlie this, including transcriptional regulation of pro-apoptotic genes, anti-apoptotic genes, signal transduction machinery, stress response machinery, etc. A potential first step in investigating this effect would be to inspect the wing disc RNA-seq datasets our lab has previously collected to identify likely candidate genes dysregulated upon Cmi/Trr KD; wing disc are superior to eye discs for this analysis due to greater homogeneity of the tissue and lack of the possibly secondary apoptotic effect. Investigation of that possibly secondary apoptotic effect in the eye disc is more difficult to approach in an un-biased fashion. My initial data

suggest that decreased Notch activity may lead to cell death in this region. This would be verified by genetic interaction experiments similar to those done with Notch pathway constructs in the adult eye, attempting to enhance, suppress, and phenocopy the apoptotic phenotype. Immunofluorescence assays would be used to measure the expression level of Notch machinery in the undifferentiated eye, similar to the previously described proposal for further investigation of the rough and shrunken eye phenotype. Any identified candidate genes associated with either the sporadic or concentrated apoptotic phenotypes would be tested in reference to the other to determine if the two are truly mechanistically separate.

The *bantam* miRNA is well-characterized as suppressor of apoptosis through translation inhibition of apoptotic gene Hid [112]. My results demonstrate that overexpression of *bantam* throughout the eye disc causes a general increase in effector caspase activation in undifferentiated eye tissue, in apparent conflict with all previous work. However, as the previous validations of *bantam*'s role in regulating cell survival focus solely within differentiating ommatidia, my work instead provides new evidence on the different roles of *bantam* activity in the undifferentiated and differentiating eye. The widespread caspase activation anterior to the morphogenetic furrow appears distinct from that caused by loss of MLR activity, suggesting different mechanisms. This effect is most likely caused by reduced expression of one or more *bantam* targets, and initial investigation would comprise of genetic interaction experiments modulating the levels of known *bantam* targets in the undifferentiated eye, identifying possible candidates through ability to enhance, suppress, or phenocopy the *bantam*-associated apoptotic effect.

The MLR Complex is Required for Regulation of Stress Response Transcription in the Fat Body

My data in the fat body suggests that the MLR complex is required to properly regulate the transcriptional activity of Foxo and/or Relish in response to stress states. If verified, this would greatly expand our comprehension of the roles of MLR complexes in terminally-differentiated cells reacting to environmental stimuli, an understudied area. To continue to investigate this, further genetic interaction experiments would be performed modulating Foxo or Relish regulation machinery (ie. Foxo, Akt, 14-3-3, Rel, Imd, Cact, etc.) in the fat body in the background of Cmi KD/OE to provide clearer evidence of association as well as to inform the likely level of regulation. The MLR complex may regulated Foxo/Relish activity at multiple levels, including transcription of Foxo/Relish, of upstream modifiers/signaling machinery, and of downstream targets. Level of regulation would also be examined by performing immunofluorescence experiments for Foxo/Relish level and localization in the fat body; changes in expression level would be interpreted as alteration of Foxo/Relish transcription, changes in localization would be interpreted as alteration of upstream regulators [197,198], and changes in neither would be interpreted as alteration of downstream targets. Whole-animal Chip-seq data would also be compared to our collected fat body RNA-seq data to correlate MLR complex localization with regulation of likely targets. Candidate genes identified by any one of these procedures would inform the design and results of the other two, leading to candidate regulatory targets of the MLR complex necessary for its role in stress response.

The fat body RNA-seq data suggests that anti-microbial response categories are among the most significantly affected by the modulation of Cmi levels, and the dysregulation of AMPs supports this. To continue to verify and supplement the investigation of MLR complex

interaction with Relish, microbial stress experiments would be performed by exposing Drosophila to pathogenic bacteria, both gram-positive (Toll pathway-specific) and gram-negative (Imd pathway-specific) and tracking survival as well as change in bacterial titer during immune response.

The MLR Complex is Required to Repress Gene Expression

In a recently published report, our lab detailed a requirement for the MLR complex to negatively regulate transcription of target genes, a novel and unexpected dimension of its function [47]. It was determined that the MLR complex plays a role in suppressing premature activation of transcriptional targets. Therefore, genes controlled by enhancers established by the MLR complex suffered two effects upon loss of the complex: aberrant upregulation before receipt of activating signal, and inability to properly respond to that signal once received. This was characterized using regulatory elements responsive to EcR, though it is unlikely that this function is unique to a particular regulatory partner of MLR. In the work presented here, I suggest more examples of negative regulatory activity requiring the MLR complex, including expression of the miRNA *bantam* in imaginal tissue, Notch activity in the developing compound eye, and Foxo activity and AMP expression in the fat body. It remains unclear whether these examples of suppression operate under similar mechanisms as EcR-response elements (premature activation), or are perhaps due to as-yet-unidentified direct negative regulatory mechanisms. Comparison of activity before and after an activating stimulus would inform this, as AMP dysregulation patterns closely mirror those found in EcR-activated genes. My results in the developing eye suggest that the MLR complex may be required to positively regulate Notch signaling in undifferentiated eye tissue, but negatively regulated it during compound eye

formation. My results in the fat body suggest that the MLR complex may be required to negatively regulate Foxo signaling in late larva, but positively regulated it during metamorphosis and in the adult. My investigation into *bantam* regulation in developing ommatidia demonstrates simultaneous opposite regulatory activity in neighboring cells of different fates. Each of these represents a unique case of MLR activity being necessary for transcription of a single target or activity of an effector that changes in regulatory "direction" depending on developmental context. The phenotypic effects of MLR complex loss in each of these cases demonstrates that this multifaceted regulatory function is necessary for development and survival. Each of these examples can also be used to further elucidate the unknown mechanisms of how the MLR complex contributes to gene suppression.

Investigation of negative regulatory activity associated with the MLR complex would require precise snapshots of the enhancer and promoter environments prior to, during, and after moments of regulatory activity. This includes a survey of bound regulatory factors, histone marks, transcription machinery, and enhancer-promoter interaction. Changes to these landscapes in response to loss of MLR complex activity would inform further directions of study. Drosophila is an ideal model organism for this investigation; in additional to its multiple benefits as a genetic and developmental model, but genetic split of *Cmi* and *trr* provides further tools in this analysis. Loss of Trr prevents complex formation while loss of Cmi allows complex formation and binding to targets yet prevents regulatory activity; however, depletion of either subunit prevents the MLR complex's suppressive role [47]. Further elucidation of these effects will undeniably be necessary for comprehending the total function of these highly-conserved epigenetic regulators as well as gene regulation as large.

The MLR Complex and Human Disease

Mutation in the two genes encoding MLR complex methyltransferases in humans, *KMT2C* and *KMT2D*, is heavily associated with disease states; germline mutation in either gives rise to development disorders and the two are among the most frequently somatically mutated in solid tumors [20]. Despite the significant association with cancer, including as potential driving mutations, it is currently unknown how alteration of MLR complex function supports oncogenesis beyond some evidence of genome instability, maintenance of multipotent state, and altered p53 activity. The results presented here suggest other possibly malignancy-promoting effects.

Drosophila cancer models have previously defined the *bantam* miRNA as harboring oncogenic potential [199]. There is no direct human ortholog to *bantam* has been identified, but two potential counterpart miRNAs have been suggested based on sequence and function. mir-450b has the greatest sequence similarity to *bantam* in the human genome [200] and has been described as suppressing cancer cell proliferation and inducing protective differentiation [201]. mir-130a has been separately described as both oncogene and tumor suppressor, impacts drug resistance [202], and is functionally orthologous to bantam in its feedback regulation of Hippo pathway signaling [127]. If human MLR complexes are also required for proper regulation of either of these, dysregulation upon MLR subunit mutation may underlie transforming events.

While Notch signaling is best associated with cell fate determination and developmental patterning, altered Notch activity has been associated with almost every hallmark of oncogenesis, particularly excess proliferation and the survival of cancer stem cells [203,204]. Dysregulation of stress signaling and inflammatory pathways, particularly those involving Foxo

and NF-κB (Relish) effectors, promote survival and metastasis of tumor cells in addition to increasing resistance to treatment [205–208]. If MLR complexes are required for proper regulation of human Notch, Foxo, and/or NF-κB transcriptional activity, either activating or suppressive, then alteration of MLR activity in cancer has clear oncogenic potential.

Further elucidation of the recruiting partners and regulatory targets of MLR complexes paves the way for therapeutic targeting. A *C. elegans* model has suggested that an MLR-like complex attenuates RAS signaling during development, and RAS/MAPK inhibitors were later successfully used to ameliorate developmental deformities in a Zebrafish model of Kabuki Syndrome [73,209]. Polycomb repressor complex 2 (PRC2) acts antagonistically against MLR complex activity; in cell lines harboring cancer-associated *KMT2C* mutations, normal gene expression patterns are restored by PRC2 inhibition [210]. The work presented here suggests further targets, such as components of developmental signaling or stress response pathways, that may prove therapeutically effective in combatting the effects of MLR complex mutation.

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VITA

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